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HPLC-ESI-DAD Analysis of Phenolic Compounds from Clematis Flammula Leaf Extract: An Assessment of Their Antioxidant Properties

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ABSTRACT:

The purpose of this study was to investigate the free-radical scavenging activity and identify the chemical composition of *Clematis flammula* leaves. The phenolic composition of *Clematis flammula* leaves from the Algerian region was investigated. Extraction was performed in ethanolic solvents. Their efficiency was determined in terms of the quantitative composition in phenolic compounds by HPLC-DAD of the extract isolated from *Clematis flammula* leaves. Its leaf extract has been found to contain a high concentration of phenolic compounds, which include trans chalcone (1.82 ± 0.18 µg/ml), 2,3-dihydroflavone (0.97 ± 0.26 µg/ml), 3-hydroxy flavone (0.85 ± 0.15 µg/ml), cinnamic acid (0.81 ± 0.11 µg/ml), catechin hydrate (0.52 ± 0.04 µg/ml), and trans hydroxy cinnamic acid (0.38 ± 0.09 µg/ml), which are the main constituents. There are also six existing minor acidic compounds and flavonoids, such as rutin hydrate, chlorogenic acid, and ellagic acid. The highest yield of phenolic compounds from *Clematis flammula* was found in the ethanolic extract. On the other hand, the ethanolic extract exhibited a high scavenging activity against DPPH inhibitory activity (IC_{50} = 7.56 µg/ml) when compared with standard BHT (IC_{50} = 0.20 µg/ml).

Keywords: *Clematis flammula*, ethanolic extract, DPPH, phenolic compounds, HPLC/DAD

1. Introduction

Clematis belongs to the Ranunculaceae family. As a wild medicinal plant, its potential pharmacological and ecological effects have attracted much attention **(Rattan, 2023; Thapliyal et al., 2024)**. The species of the genus Clematis emerged as a good source of traditional medicine for the treatment of various ailments. Although few experimental studies validated their traditional claims, they employed uncharacterized crude extracts **(Chawla et al., 2012; Rattan, 2023)**. It used to cultivate medicinal plants in southern Europe, China, and North Africa **(Wu et al., 2001)** . However, it is mainly distributed around the Mediterranean area and is very common in hedges and woods. In Algeria, *Clematis flammula* is a perennial climbing plant that grows naturally in the highlands of the Kabylie region in the Bejaia Province **(Beloued, 1998)**. Its considerable chemical composition and biological activities, pharmacological actions are also interesting for the scientific circle **(Atmani et al., 2009; Belkhir et al., 2024)**. A number of studies have been carried out concerning its chemical

composition and popular ways of application, such as doubtless due to their antioxidant activity **(Atmani et al., 2009; Saidi et al., 2019)**, cytotoxic **(Atmani et al., 2011),** neuroprotection, anti-inflammation, anti-cancer potentials **(Medjahed et al., 2023)**, anti-geno-toxicity, and others studies. Bioactive compounds responsible in the plant include triterpenoid saponins, flavonoids, coumarins, and alkaloids, as well as volatile oils, and offer potential drug development **(Hao et al., 2013)**. However, the bioavailability of these compounds, particularly after simulated human digestion, needs further investigation. These properties are attributed to its phenolic contents and flavonoids general antioxidant activity. Bioactive principles and potential drugs from the species need more investigations **(Acar Şah et al., 2019; Tebbi et al., 2022)**. According to epidemiological studies, infusions prepared from dried leaves are used to treat many ailments, specifically arthritis, superficial burns, and even cancer. Moreover, whole leaves are used as an insect repellent against deterioration of stored wheat **(Atmani et al., 2011)**.These studies collectively propose the potential of *Clematis flammula* in the pharmaceutical and healthcare industries, but moreover highlight the need for comprehensive investigation to completely get its benefits. The display inquiry was carried out to explore the composition of the polyphenol division utilizing HPLC-DAD, the quantification of phenols has been determined in order to correlate antioxidant activities tested. This ponder clearly supports the phytochemical potential of *Clematis flammula* and led one step further to the development of a new drug that is nutraceuticals or dietary food supplements prepared for health-conscious people.

2. Material and methods

2.1. Plant material

Our study was carried out on the leaves of *Clematis flammua*, which were harvested in May 2023 from the forest in Amizour, Bejaia province in Northeastern Algeria. Identification of the investigated specie was confirmed in the herbarium of Botany Department, National Higher Normal School of kouba (ENSK) by Pr. Mohamed Toumi, (University of Algeria). The voucher specimens were deposited in a botanical collection of ENSK herbarium (CB05062021). The samples of leaves were dried in a well-ventilated area to eliminate any trace of moisture while preserving the composition of the plant. They were then ground using an electric grinder and sieved to recover a fine powder.

2.2. Extraction of bioactive substances

The extraction was carried out according to the method of **(Atmani et al., 2009)** with some modifications. The plant powder was macerated in ethanol (96%. v/v) with a ratio of 1/4, (m/v) for 48 hours under agitation. After decantation for 24 hours, the supernatant was recovered in crystallizers and left to dry in the open air until a dry residue of stable weight was obtained. The extracts were stored in airtight glass bottles at -4°C to preserve the active principles of the plant.

2.3. Chemical standards

2.3.1. Reagents and Solvents

Solvent ethanol (99.9 % purity), was purchased from Sigma-Aldrich Chemie (St. Louis, MO, USA). The following phenolic compounds were purchased from Sigma-Aldrich Chemie (St. Louis, MO, USA): ascorbic acid, orcinol, catechin hydrate, trans-hydroxy cinnamic acid, rutin hydrate, naringenin, kaempferol, 3-hydroxy flavone, flavanone, ferulic acid, coumaric acid, cinnamic acid, gallic acid, syringic acid, salicylic acid, ellagic acid, chlorogénic acid, epigallocatechin gallate, quercetin, p-hydroxy benzoic acid, Trans chalcone, sinapic acid. The standard solutions were diluted in ethanol solvent (standard dilutions: 1 mg/mL) and injected in triplicate.

2.3.2. DPPH Free-radical scavenging Activity

The DPPH radical scavenging activity was determined in accordance with the methodology described by **(Bicas et al., 2011)** , with some modifications. Briefly, 0.5 mL of the ethanolic extract at varying concentrations (25, 50, 75 and 100 µg/mL) was combined with 0.5 mL of ethanolic DPPH solution (0.004 % w/v). The mixture was incubated at room temperature for 30 minutes, after which the absorbance was measured at 517 nanometers using an ultrospec 7000 UV-vis dual beam spectrophotometer (GE Healthcare, Chicago, IL, USA). Butyl hydroxy toluene was used as a positive standard. The inhibition percentage of the DPPH free radical (I %) was calculated using the following formula:

I% = [(Ab - Aa)/Ab]×100.

All tests were performed in triplicate. Where, Ab is the absorbance of the reaction media without extract and Aa is the absorbance of the test sample.

2.3.3. Identification of peaks and peak purity

All constituents were identified by HPLC-DAD analysis by comparing their retention time, UV spectra with those of authentic reference samples. The purity of each peak was checked using a Diode Array Detector coupled to the HPLC system **(Rezig et al., 2024)**.

2.3.4. HPLC-DAD analysis

The ethanolic extract was dissolved in ethanolic solution: (10: 96 v/v) before injected to HPLC. Twenty-three phenolic compound standards were analyzed and identified using HPLC. The diluted samples from *C. flammula* leaves were injected to HPLC-DAD. Phenolic compounds separation was performed with an Agilent 1260 series HPLC system equipped with quaternary pump with degasser (G1311B) associated with a thermostatic auto sampler (G 1329 B). Instrument control and data analysis were carried out using Agilent HPLC chemstation (B 04.01). Phenolic compounds separation was carried out on a reverse phase C18 column (5 µm, 150 mm×3 mm i.d) thermos stated at 35 °C by HPLC equipped with a diode array detector. The solvent system used was a gradient of A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile) according to the method described by **(Bakhouche et al., 2013)** with minor modification. The solvent flow rate was 1.5 mL/min. The sample volume injection was 20 µL. The elution gradient was as follows: 0–5 % B (0–10 min); 5–17 % B (10–18 min);17–25% B (18–33 min); 25–100 % B (33–48 min); 100 – 5 % B (48–51 min). Detection and the identification of phenolic compounds were carried out at 254.4 nm using a diode array detector (DAD); peak identification was obtained comparing the retention time and the UV spectra of the chromatogram with those of pure standards.

3. Results and discussion

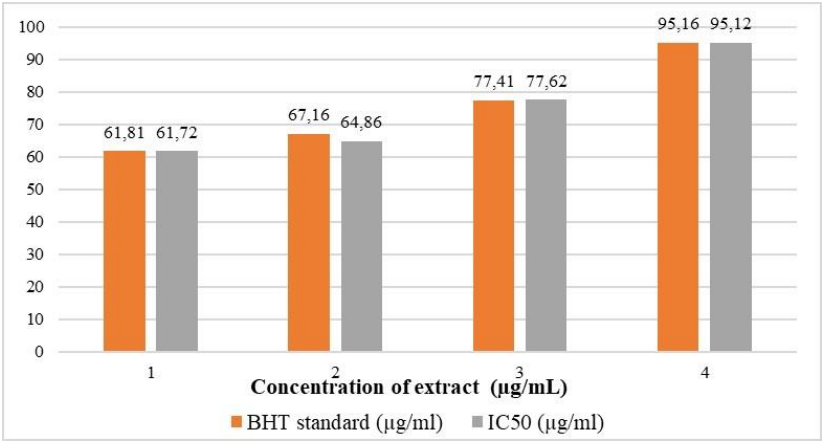

Figure 1.The scavenging activity of phenolic compounds extricated from leaves of C. flammula according to different concentration

The scavenging activity of the extract from the plant against the stable radical (DPPH) has been evaluated at concentration of 25 to 100 µg/ml; the results are illustrated in "Figure 1". Concerning *clematis flammula*, the ethanolic extract exhibited the best outstanding DPPH scavenging activity (IC_{50} = 61.72 µg/ml, 95.12 µg/ml, respectively), significantly lower than that of BHT (61.81 µg/ml, 95.16 µg/ml). Further studies using varying concentrations (25 to 100 µg/ml) of extracts from our plant were also carried out. In the case of *clematis flammula*, our results are supported by the fact that ethanol extract from Algeria *Clematis flammula* exhibited a DPPH scavenging activity of more than 90% at 100 µg/ml

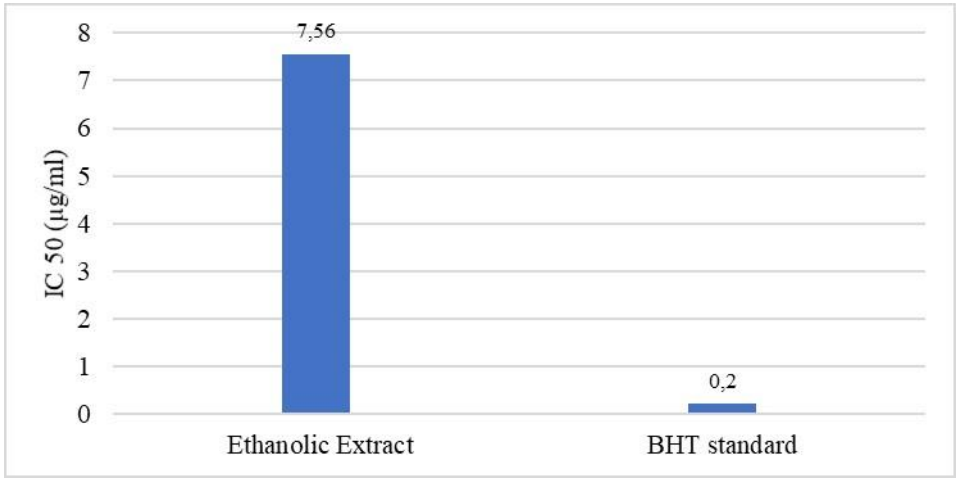

Figure 2. DPPH Free-radical scavenging activity of C. flammula extract

The scavenging activity of *Clematis flammula* extract obtained from selective ethanolic extract by studying in vitro "Figure 2". The ethanolic extract showed high antioxidant activity against the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) (IC_{50} = 7.56 µg/ml) when compared with standard BHT (IC_{50} = 0.20 µg/ml), Previous result of **(Chohra et al., 2020)** confirmed also that *Clematis flammula* extracts have scavenging activity by obtaining results for other extract in same plant, such as methanolic and ethanolic extracts. Moreover, other research found the ethanol extract of *Clematis flammula* demonstrated a scavenging activity of 34% against superoxide, and was very effective against the hydroxyl radical (61%) at 100 µg/ml, in the same previous research, both considered as important initiators of lipid peroxidation **(Atmani et al., 2011)**. However, this same study indicated that the ethanol extract of *Clematis flammula* exhibited a low inhibition capacity of (80%) at 1 µg/ml against lip peroxidation of microsomes compared to the caffeic acid **(Atmani et al., 2011; Belkhir et al., 2024)**. On the other hand, even though the aqueous fraction issued from chloroform of *Clematis flammula* showed a scavenging activity that is almost as high as that of BHA at 100 µg/ml, its IC_{50} is relatively high (25.02 µg/ml), suggesting that its scavenging potential against DPPH is moderate. Even though the different fractions of C. flammula have previously been tested for their total antioxidant capacity using the DPPH test **(Atmani et al., 2011)**, more than one method is necessary to evaluate the antioxidant capacity of a substance or plant extract since the underlying mechanisms can be different for each test **(Frankel, 2001)**.

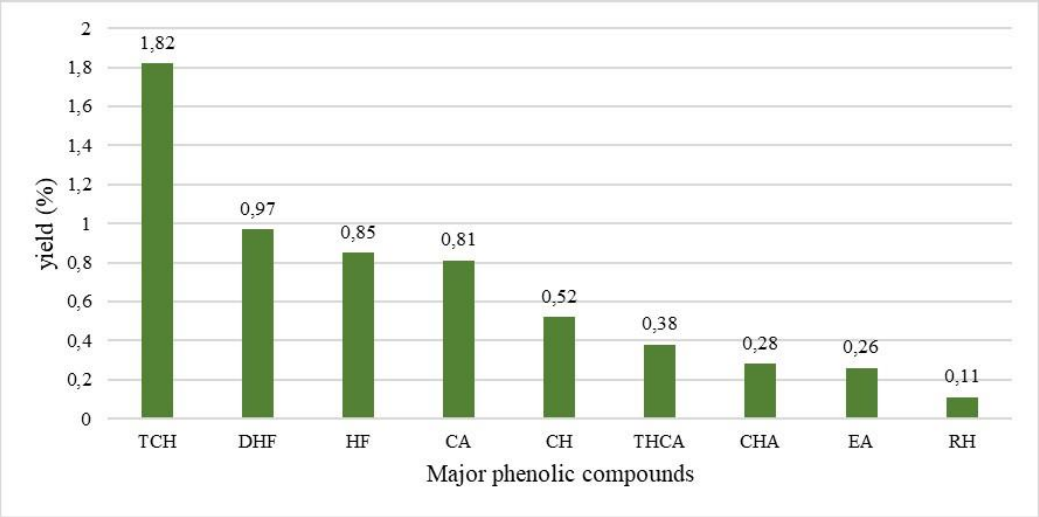

Figure 3. Phenolic compounds identified by HPLC-DAD

TCH: Trans chalcone, DHF: 2,3-Dihydroflavone HF: 3-Hydroxy flavone, CA: Cinnamic acid, CH: Catechin hydrate, THCA: Trans hydroxy cinnamic acid, CHA: Chlorogenic acid, EA: Ellagic acid, RH: Rutin hydrate

The ethanolic extract leaves of *Clematis flammula* contains several of major phenolic compounds "Figure 3" including Trans chalcone (1.82 ± 0.18 mg/g), 2,3-dihydroflavone (0.97 ± 0.26 mg/g), 3-hydroxy flavone (0.85 ± 0.15 mg/g), cinnamic acid (0.81 ± 0.11 mg/g), catechin hydrate (0.52 ± 0.04 mg/g), trans hydroxy cinnamic acid (0.38 ± 0.09 mg/g), chlorogenic acid (0.28 ± 0.06 mg/g), ellagic acid (0.26 ± 0.05 mg/g), and rutin hydrate (0.11 ± 0.01 mg/g) .These results are consistent with other studies that have reported the presence of various phenolic compounds in Clematis species. For example, a study by **(Saidi et al., 2019)** identified two flavonoids-glucoside and trisaccharide in *C.flammula* extracts. Another study found that *C. flammula* contains phenolic profiles with antioxidant and enzyme inhibitory effects **(Alruwad et al., 2024)**. Additionally, studies of **(Atmani et al., 2009)** and **(Chawla et al., 2012)** have reported that Clematis species are rich in polyphenols, flavonoids, alkaloids, and other bioactive compounds .These compounds have been implicated in hepatoprotective effects through antioxidant activity and induction of antioxidant enzymes, such as superoxide dismutase (SOD) **(Atmani et al., 2009)**.
These results are consistent with the previous HPLC analysis of *Clematis flammula* leaves, which also identified phenolic compounds such as flavonoids, phenolic acids, and chalcones in the plant extract. The differences in the specific compounds detected and their relative abundances may be due to variations in the extraction methods, plant material, and geographical origin **(Bellik et al., 2021; Rezig et al., 2024)**. Additionally, phenolic compounds have been studied for their potential anti-inflammatory effects **(Ambriz-Pérez et al., 2016),** making them valuable in combating inflammation-related conditions. In traditional medicine, *Clematis flammula* rich in phenolic compounds have been used for their therapeutic benefits, it including treating rheumatoid arthritis **(Tebbi et al., 2022; Medjahed et al., 2023)**, burns, and superficial wounds **(Saidi et al., 2018)**. Research on *Clematis flammula* has revealed a rich composition of polyphenols with various biological and therapeutic effects **(Medjahed et al., 2023)**. These compounds, including flavonoids, phenolic acids, and chalcone, are key components of Clematis plants. Studies have shown that phenolic compounds contribute to the mechanical resistance of cell walls, influence color, flavor, and aroma in plants, and have therapeutic properties such as antioxidant and anti-inflammatory effects. The presence of specific phenolic compounds like gallic acid (0.03 ± 0.007), catechin hydrate (0.52 ± 0.04), chlorogenic acid (0.28 ± 0.06), cinnamic acid (0.81 ± 0.11), and others in *Clematis flammula* indicates its potential health benefits **(Acar Şah et**

al., 2019; Alruwad et al., 2024; Bakhouche et al., 2013). Previous result of **(Medjahed et al., 2023)** and **(Letsiou et al., 2023)** confirmed our work finding some phenolic compounds such as catechin (50 ± 0.21 ug/L), kaempferol (1 ± 0.10 ug/L), rutin (10 ± 0.01 ug/L), ascorbic acid (1 ± 0.02 ug/L), caffeic acid (3 ± 0.01 ug/L), cinnamic acid (3 ± 0.02 ug/L), gallic acid (3 ± 0.01 ug/L). These phenolic compounds may contribute to the plant's medicinal properties and could be explored for their pharmacological activities **(Zhang et al., 2011)** including antioxidant, anti-inflammatory, and cytotoxic effects. Overall, the phenolic compounds in *Clematis flammula* highlight its therapeutic potential and the importance of further research to fully understand and harness the benefits of these bioactive compounds.

Conclusion and perspectives

In conclusion, this research on *Clematis flammula* has provided insights into the chemical composition aspect of this plant species. Through our experimental studies, we have unraveled the chemical composition of *Clematis flammula* and its potential medicinal properties.

Analysis of the polyphenolic composition of simple from region revealed a moderate difference in the percentage of compounds using HPLC. This variation in composition had a clear impact on the antioxidant properties of the sample.

Additionally, this study has shed light on overall, the findings from scientific journals underscore the significance of continuing research on *Clematis flammula* to further explore its potential applications in medicine and conservation.

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