Identification, Quantification, and Antioxidant Activity of Hydroalcoholic Extract of *Artemisia campestris* from Algeria

Cezayir’de Yetişen *Artemisia campestris*’in Sulu Alkollü Ekstresinin Tanımlanması, Kantitasyonu ve Antioksidan Aktivitesi

**ABSTRACT**

**Objectives:** Our study aimed to investigate the chemical profile of hydroalcoholic extract of Algerian *Artemisia campestris* and its antioxidant activity.

**Materials and Methods:** The hydroalcoholic extract of Algerian *A. campestris* was investigated for its phenolic constituents using high performance liquid chromatography (HPLC)-diode array detection (DAD)-electrospray ionization (ESI)-mass spectrometer (MS)/MS. The *in vitro* antioxidant activity and total phenolic content were also evaluated via oxygen radical absorbance capacity and Folin-Ciocalteu assays, respectively.

**Results:** HPLC-DAD-ESI-MS/MS analysis revealed that the main tentatively identified compounds were caffeoylquinic acid isomers, flavonoids, and benzoic acid derivatives. Additionally, the hydroalcoholic extract exhibited a promising antioxidant activity value of 120.5±10.4 μmol Trolox equivalent antioxidant capacity/g dry weight (DW), and a strong correlation exists between this activity and the total phenolic content value of 102.09±1.65 mg/g gallic acid equivalents DW.

**Conclusion:** The hydroalcoholic extract of *A. campestris* is a promising candidate for the production of naturally occurring antioxidant agents.

**Key words:** *Artemisia campestris*, polyphenols, flavonoids, chlorogenic acid, antioxidant

**ÖZ**

Amaç: Bu çalışmada, Cezayir’de yetişen *Artemisia campestris*’in sulu alkollü ekstresinin kimyasal profilinin ve antioksidan etkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Cezayir’de yetişen *A. campestris*’in sulu alkollü ekstresinin fenolik bileşenleri yüksek performanslı sivi kromatografisi (HPLC) diyet dizini dedektör-(DAD)-elektrospray ionizasyon (ESI)-mass spektrometresi (MS)/MS kullanılarak incelenmiştir. *In vitro* antioksidan aktivite ve toplam fenolik içerik testi de sırasıyla oksijen radikal absorbans kapasitesi ve Folin-Ciocalteu analizleri ile değerlendirilmiştir.

Bulgular: HPLC-DAD-ESI-MS/MS analizi, esas olarak saptanan ana bileşiklerin, kafeoilkuinik asit izomerleri, flavonoitler ve benzoik asit türevleri olduğu ortaya koymustur. Bununla birlikte, sulu alkollü ekstre 120.5±10.4 μmol Trolox eş değeri antioksidan kapasitesi/g kuru ağırlık (KA) değeri ile önemli derecede antioksidan aktivite göstermiş ve bu aktivite ile 102.09±1.65 mg/g gallic asit eş değeri KA olduğu belirlenen toplam fenolik içerik değeri arasında güçlü bir korelasyon saptanmıştır.

Sonuç: Sonuç olarak, *A. campestris*’in sulu alkollü ekstresinin, doğal antioksidan ajanların üretimi için umut verici bir aday olduğu belirtilmiştir.

Anahtar kelimeler: *Artemisia campestris*, polifenoller, flavonoitler, klorojenik asit, antioksidan

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Received: 01.02.2018, Accepted: 25.04.2018*
INTRODUCTION

The genus *Artemisia* is one of the largest and most widely distributed genera of the family *Asteraceae* in Europe and North Africa, and its species have been characterized for their pronounced biological activities and are considered to produce most medicinally important secondary metabolites. Eleven species of *Artemisia* can be found in the Algerian flora.1,2 *Artemisia campestris* is a perennial faintly aromatic herb widespread in the south of Algeria, commonly known as “dgouft”. The aerial parts of the plant have been used in traditional medicine as a febrifuge, vermifuge, and anticancer agent and to treat digestive troubles, gastric ulcer, and menstrual pain.3,5 A. *campestris* extract was reported to be a potent free radical scavenger of 2,2’-diphenyl-1-picryl hydrazyl, 2,2’-azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS•−), and superoxide anion radicals (O2•−) but there is a lack of knowledge regarding the phenolic composition of this plant and its relation with its antioxidant properties, since only a few studies have identified a small number of phenolic compounds.6,8

However, the phenolic profile of *A. campestris* is quite complex. Flavonoids present in this species consist of flavones, flavonols, flavanones, dihydroflavonols, and their methyl ethers, whereas the isolation of coumarins and phloracetophenones is also reported.9,10 Chlorogenic acid is a natural product occurring in a large number of different plants or parts of the plant; for example, in *A. campestris* chemically it is the ester of caffeic acid and quinic acid, 3-O-caffeoylquinic acid. Other isomers are derivative chlorogenic acid 4-O-caffeoylquinic acid and 5-O-caffeoylquinic acid. Additionally, there are other isomers, called iso-chlorogenic acids, with two caffeic acid moieties such as 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, and 1,5-dicaffeoylquinic acid.

The objective of the present work was to contribute to the identification of the major phenolic compounds in the hydroalcoholic extract of *A. campestris* by high-performance liquid chromatographic/diode array detector (HPLC-DAD) coupled with electrospray ionization/mass spectrometry (ESI-MS). In addition, HPLC-DAD-electrochemical detector quantification of phenolic and flavonoid contents and hydroxycinnamic acid was carried out. Finally, the antioxidant capacity of the extract was also evaluated by oxygen radical absorbance capacity (ORAC) assay.

EXPERIMENTS

**Chemicals**

Chlorogenic acid was purchased from Extrasynthese (Genay, France). Methanol for HPLC-GOLD-Ultra gradient was purchased from Carlo Erba Reagents (Val de Reuil, France). Phosphoric acid (85%) and formic acid (98%) were purchased from Panreac Química (Barcelona, Spain). Acetonitrile HPLC gradient grade was purchased from VWR® (Leuven, Belgium). Milli-Q® water (18.2 MΩ·cm) was obtained in a Millipore-Direct Q3 ultraviolet (UV) System (Molsheim, France).

**Plant material**

Aerial parts of *A. campestris* were collected from the Laghouat region in the northern Algerian Sahara in summer 2015. The identification and authentication of the plant were carried out by Dr. Mohamed Koundri, botanist (Department of Agronomy, Faculty of Sciences, University of Laghouat, Algeria) and the voucher specimens were deposited at the Laboratory of Process Engineering, University of Laghouat (number LGP Ac/08/15).

**Preparation of the hydroalcoholic extract**

One gram of dried powder was mixed with ethanol:water (8:2; v/v, 10 mL) and macerated under sonication, (water bath, room temperature, 30 min). The material was filtered and the crude extract obtained was analyzed directly by HPLC. The procedure was performed in triplicate.

**Equipment and conditions of analysis**

**Liquid chromatography with diode array and electrochemical detection**

The HPLC system used was a Thermo Finnigan (Surveyor, San Jose, CA, USA), equipped with an autosampler, pump, photodiode-array detector (PDA), and electrochemical detector (ED). Chromatographic separation of compounds was carried out on a Lichrocart RP-18 column (250×4 mm, particle size 5 μm, Merck). The Dionex® ED performed signal measurements by integrated voltammetry at potentials between -1.0 V and 1.0 V with a scan time of 1.00 s. The obtained results were acquired at a frequency of 50 Hz using an analogue/digital converter. The photodiode array detector was programmed for scanning between 192 and 798 nm at a speed of 1 Hz with a bandwidth of 5 nm. The detection was monitored using three individual channels, 280, 320, and 360 nm, at a speed of 10 Hz with a bandwidth of 11 nm. The injection volume was 20.00 μL and total time of analysis was 120 min. A binary gradient elution (Table 1) was used. The mobile phase was as follows: 0.5% formic acid in Milli-Q® Water 95% (eluent A) and 0.5% phosphoric acid in acetonitrile 90% and 9.5% Milli-Q® Water (eluent B). The flow rate was systematically controlled and set at 0.3 mL/min.

**Liquid chromatography with mass spectrometry**

The identification of compounds in the extracts was carried out by HPLC-MS/MS using Waters® Alliance 2695 HPLC equipment fitted with a DAD, Waters 2996 (PDA), and a triple quadrupole spectrometer (TO) (Micromass® Quattro micro™, Waters) with an ESI source operating in negative mode. The capillary in the ESI source was placed at 3.0 kV and the cone at 30 V. The chromatographic separation was performed on a LiChroCART RP-18 column (250×4 mm, particularly from size 5 μm, Merck) at 35°C. The eluents used were A: formic acid (0.5% v/v) and B: acetonitrile (LC-MS grade). A gradient elution program was applied for chromatographic analysis (Table 1). Flow rate was maintained at 0.3 mL/min and the injection volume was 10 μL. Ultrapure nitrogen (N2) was used as nebulizer and drying gas and gas. Ultrapure argon was used as the collision gas at a
pressure of 10⁻⁴ mbar. For data acquisition and treatment of data MassLynx® software version 4.1 was used.

**Determination of phenolic chromatographic profile**

Total phenolic content was determined using the 280 nm total peak area above 40 min. Calibration curves with gallic acid (0-25 ppm) were created and the final results were expressed in terms of gallic acid equivalents (GAE) per gram of dry weight (DW) (mg/g GAE DW).

Total flavonoids content was determined using the 360 nm total peak area above 40 min. Calibration curves with rutin (0-50 ppm) were created and the final results were expressed in terms of rutin equivalents (RE) per gram of DW (mg/g RE DW).

Total hydroxycinnamic acids content was determined using the 320 nm total peak area between 20 and 40 min. Calibration curves with caffeic acid (0-25 ppm) were created and the final results were expressed in terms of caffeic acid (CA) equivalents per gram of DW (mg/g CA DW). Additionally, the content of total phenols was determined colorimetrically with Folin’s reagent according to the method reported by Stamatakis et al.¹¹ The phenolic contents were expressed as mg of GAE per gram of DW (mg/g GAE DW).

**ORAC**

Peroxyl radical scavenging capacity was determined by the ORAC method. The assay was carried out by following the method reported by Huang et al.¹² modified for the FL800 microplate reader (BioTek Instruments, Winooski, VT, USA) as described by Feliciano et al.¹³ All data were expressed as micromoles of Trolox equivalent antioxidant capacity (TEAC) per gram DW (μmol TEAC/g DW).

**RESULTS**

The HPLC method employed for the separation of phenolic components in the hydroalcoholic extract of *A. campestris* revealed a good separation of the majority of the compounds. Chromatograms at 280 nm are widely used to study phenolic compounds because absorption at this wavelength is suitable to detect a large number of such compounds. The maximum absorption wavelengths (λ<sub>max</sub>), and parent, aglycone, and fragment ion masses of the components detected in the aqueous extract of *A. campestris* are shown in Table 2, where the compounds are numbered according to their retention times (R<sub>t</sub>) in the obtained chromatograms.

Four compounds were unequivocally identified based on the analysis of standard compounds and comparing their HPLC retention time, UV spectra, and MS/MS fragmentation pattern. The remaining compounds were characterized and their structures proposed based mainly on the MS/MS fragmentation data conjugated with the UV-DAD spectra. Most of the peaks showed similar UV absorptions maxima with two bands at λ<sub>max</sub> 230-240 nm and 320-330 nm. These types of UV absorption bands are characteristic of hydroxycinnamic acids. Some peaks with characteristic UV absorptions bands for flavonoids were

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**Table 1. Gradient eluents used for analysis by HPLC-DAD-ED**

| Time (min) | Eluent A (%) | Eluent B (%) |
|------------|--------------|--------------|
| 0.10       | 98.90        | 1.10         |
| 15         | 91.00        | 9.00         |
| 20         | 87.80        | 12.20        |
| 30         | 87.80        | 12.20        |
| 55         | 86.50        | 13.50        |
| 95         | 73.00        | 27.00        |
| 105        | 37.00        | 63.00        |
| 110        | 37.00        | 63.00        |
| 125        | 98.90        | 1.10         |
| 130        | 98.90        | 1.10         |

HPLC: High performance liquid chromatography, DAD: Diode array detection, ED: Electrochemical detector

**Table 2. Phenolic compounds tentatively identified in hydroalcoholic extract of Artemisia campestris**

| Peak no. | R<sub>t</sub> (min) | Ultraviolet | (M-H) m/z | Fragmentations | Compounds proposed | References |
|----------|---------------------|-------------|-----------|----------------|-------------------|------------|
| 1        | 27.5                | 259         | 153       | 141, 109       | Protocatechuic acid | 19         |
| 2        | 29.45               | 325         | 353       | 191, 179, 173  | 5-O-Caffeoylquinic acid | 28         |
| 3        | 31.6                | 266         | 205       | 143, 129, 114  | Quinic acid methyl ester | 20         |
| 4        | 40.26               | 224/326     | 353       | 191, 173, 85   | 3-O-Caffeoylquinic acid | 28         |
| 5        | 44.9                | 325         | 179       | 135, 107, 89   | Caffeic acid       | 21         |
| 6        | 58.6                | 328         | 367       | 191, 173, 134, 93, 87 | 4-O-Feruloylquinic acid | 22         |
| 7        | 73.3                | 365         | 463       | 301, 179, 151  | Quercetin-O-glucoside | 23         |
| 8        | 76.8                | 256         | 609       | 301            | Rutin              | 24         |
| 9        | 83.68               | 247/326     | 515       | 353, 235,191, 179, 173, 135 | 3,4-Dicaffeoylquinic acid | 28         |
| 10       | 89.18               | 244/326     | 515       | 353, 191, 179, 173, 135 | 4,5-Dicaffeoylquinic acid | 28         |
| 11       | 114.0               | 313         | 298, 283, 255, 163, 117 | 4',7'-Dimethoxy luteolin | 25         |
also detected. The chromatogram of the hydroalcoholic extract of the aerial parts from *A. campestris* is presented in Figure 1. The most relevant components were caffeoylquinic acids. In general, in the MS spectrum the most intense peak corresponds to the deprotonated molecular ion [M-H]−. The main fragments observed in the MS/MS experiments are given in Table 2. Chemical structures of some phenolic compounds tentatively identified in hydroalcoholic extract of *Artemisia campestris* are given in Figure 2.

**Quantification of chlorogenic acid derivatives of *A. campestris***

The content of chlorogenic acid derivatives of *A. campestris* extract was determined. The amounts of the identified compounds are given in Table 3. 3,4-Dicaffeoylquinic acid was the major caffeoylquinic acid in the hydroalcoholic extract of *A. campestris* (274.7±9.50 mg eq Trolox/L).

The data in Table 3 reveal the highest quantities of the three isomers of the caffeoylquinic acid (3-caffeoylquinic acid) and 5-caffeoylquinic acid in the hydroalcoholic extract of *A. campestris*.

**Antioxidant activity and total phenolic content**

The antioxidant and total phenolic content of the *A. campestris* extract were measured by ORAC assay and the results are shown in Table 4.

**DISCUSSION**

**Characterization of caffeoylquinic acids (M=354) and dicafeoylquinic acids (M=516)**

Two peaks were detected at m/z 353 and assigned using the hierarchical keys previously developed as well-known chlorogenic acid (3-O-cafeoylquinic acid) and 5-O-cafeoylquinic acid. Two dicafeoylquinic acid isomers were identified by their parent ion m/z 515 and were assigned as 3,4-dicafeoylquinic acid and 4,5-dicafeoylquinic acid.

**Characterization of other nuclei**

A peak was detected at *R*<sub>t</sub>=27.5 min with [M-H]− at m/z 153 with a characteristic MS<sup>2</sup> fragment at m/z 109 [M-H-44]− due to loss of CO<sub>2</sub> moiety; it was identified as 3,4-dihydroxybenzoic acid (protocatechuic acid). Another peak at *R*<sub>t</sub>=31.6 min showed a deprotonated molecule [M-H]− at m/z 205 with MS<sup>2</sup> fragments of 143, 129, and 114; it was assigned to quinic acid methyl ester.

A molecular ion was seen at *R*<sub>t</sub>=44.9 with a deprotonated ion [M-H]− at m/z 179 with daughter ions at m/z 135 [M-H-44]− due to the neutral loss of CO moiety and 107 [M-H-44-28]− due to further neutral loss of CO moiety; it was identified as 3,4-dihydroxy-cinnamic acid (caffeic acid) as previously described.

A peak at *R*<sub>t</sub>=58.6 showed a deprotonated ion [M-H]− at m/z 367 and MS<sup>2</sup> ions at m/z 191 equivalent to quinic acid moiety, and another fragment at m/z 173 due to loss of H<sub>2</sub>O molecule; it was identified as 4-O-feruloylquinic acid. Moreover, a peak at *R*<sub>t</sub>=73.3 showed a deprotonated ion [M-H]− at m/z 463 and MS<sup>2</sup> ions at m/z 301 due to loss of glucose moiety (-m/z 162) and equivalent to quercetin aglycone moiety. In addition, characteristic fragments of aglycone appeared at m/z 179 and 151; it was identified as quercetin-O-glucoside.

A peak at *R*<sub>t</sub>=76.8 showed a deprotonated ion [M-H]− at m/z 609 and a characteristic MS<sup>2</sup> ion at m/z 301 due to loss of rutinosyl moiety (-m/z 308) and equivalent to quercetin aglycone moiety; it was identified as quercetin-3-O-rutinoside (rutin).

Table 3. Quantification of chlorogenic acid derivatives by electrochemical detector

| Peak no. | *R*<sub>t</sub> | m/z | Compounds proposed            | mg eq Trolox/L |
|----------|----------------|-----|-------------------------------|----------------|
| 2        | 29.45          | 353 | 5-O-Caffeoylquinic acid       | 6.48±0.25      |
| 4        | 40.26          | 353 | Chlorogenic acid (3-O-Caffeoylquinic acid) | 191.92±5.4 |
| 9        | 83.68      | 515 | 3,4-Dicaffeoylquinic acid     | 274.7±9.50    |
| 10       | 89.18       | 515 | 4,5-Dicaffeoylquinic acid     | 117.6±3.52    |

**Figure 1.** Chromatographic profile of *Artemisia campestris* obtained by HPLC-DAD at 280 nm

HPLC: High performance liquid chromatography, DAD: Diode array detection

Table 4. Phenolic, hydroxycinnamic acid, and flavonoids contents and value of ORAC assay of *Artemisia campestris* extract

| Sample                  | TPC (280 nm) mg/g DW | HAC (320 nm) mg/g CA DW | TFC (360 nm) mg/g RE DW | TPC (Folin method) mg/g EGA DW | ORAC μmol TEAC/ g DW |
|-------------------------|----------------------|------------------------|------------------------|-------------------------------|-----------------------|
| *Artemisia campestris* extract | 61.4±2.13            | 37.26±0.88             | 17.94±1.26             | 102.09±1.65                   | 120.5±10.4            |

TPC: Total phenolic content, HAC: Hydroxycinnamic acid content, TFC: Total flavonoids content, CA: Caffeic acid, DW: Dry weight, RE: Rutin equivalents, EGA: Equivalents of gallic acid, TEAC: Trolox equivalent antioxidant capacity, ORAC: Oxygen radical absorbance capacity
a peak at $R_t=114.0$ showed a deprotonated ion $[\text{M-H}]^-$ at $m/z$ 313 and characteristic MS$^n$ ions at $m/z$ 298 due to the loss of methyl moiety $[\text{M-H-CH}_3]^-$ and 283 due to further loss of another methyl moiety $[\text{M-H-2CH}_3]^-$; it was identified as 4$^\prime$,7$^\prime$-dimethoxy luteolin.25

**Antioxidant activity and total phenolic content**

In the current study, the ORAC (Trolox equivalents, TE) value (120.5±10.4 μmol TEAC/g DW) was below the results (263.65±39.7 μmol TEAC/g DW) found by Bakchiche et al.7 and higher than the values of different *Artemisia* species harvested in Korea reported by Lee.26 This can be due to several reasons such as the method of extraction and the date and place of harvest (seasonal variations).

The reagent Folin–Ciocalteu is used in the quantification of total phenols; it is not only specific for phenols but also has the ability to react with sugar, protein, etc. For this reason our result was very high. We found a value greater than the values of the total phenols with the same species reported by Djeridane et al.27 (20.38 mg/g GAE DW) and Bakchiche et al.7 (53.84 mg/g GAE DW).

Bakchiche et al.7 previously stated that the hydroalcoholic extract from aerial parts of *A. campestris* possessed high antioxidant activity coupled to high phenolic content. Further investigation of known phenolic compounds in this extract, quantified by HPLC-MS/MS, revealed that chlorogenic acid was in high abundance (161.9±5.4 mg/g DW) and was most likely responsible for the majority of the observed antioxidant activity.7 In the current study, *A. campestris* extract, which demonstrated high antioxidant activity and phenolic content, was further analyzed for the presence of a number of mono (3-O-cafeoylquinic, 5-O-cffeoylquinicacids) and di (3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid) substituted chlorogenic acid derivatives using HPLC-MS/MS. Numerous previous reports revealed the antioxidant activity of medicinal plants based on the presence of certain polyphenolic compounds including phenolic acids, flavonoids, tannins, and their derivatives.28-30

**CONCLUSIONS**

The aim of the present study was to contribute to the identification of the major phenolic compounds in the hydroalcoholic extract of *A. campestris*; quantification of phenolic and flavonoid contents and hydroxycinnamic acid was carried out, and the antioxidant capacity of the extract was evaluated by ORAC assay. According to the data obtained, 11 phenolic compounds in the hydroalcoholic extract were tentatively identified using HPLC-DAD-ESI-MS/MS. The identified compounds contained phenolic acid derivatives and flavonoids. Moreover, the hydroalcoholic extract showed a noticeable antioxidant potential; this high activity may be due to the presence of phenolic compounds. In conclusion, the aerial parts of *A. campestris* are considered a promising source of naturally occurring antioxidant agents, and its polyphenol profile may be regarded as a model for caffeoylquinic acid distribution in the plant *A. campestris* and can help to distinguish chlorogenic acid isomers.

Conflict of Interest: No conflict of interest was declared by the authors.

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