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Identification, Quantification and Antioxidant Activity of Hydro-alcoholic Extractof *Artemisia campestris* from Algeria

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ABSTRACT

In this study, the hydro-alcoholic extract of Algerian*Artemisia campestris* was investigated for its phenolic constituents using HPLC-DAD-ESI-MS/MS. Also, the *in vitro* antioxidant activity and total phenolic content were evaluated via ORAC and Folin Ciocalteu assays, respectively. HPLC-DAD-ESI-MS/MS analysis revealed that the main tentatively identified compounds are caffeoylquinic acid isomers, flavonoids and benzoic acid derivatives. Additionally, the hydroalcoholic extract exhibited promising antioxidant activity value of 120.5 ± 10.4 µmol TEAC/gDW, and a strong correlation exists between thisactivity and the total phenolic contentvalue of 102.09 ± 1.65mg/g GAE DW. In conclusion, the hydro-alcoholic extract of *Artemisia campestris*is a promising candidate for the production of naturally occurring antioxidant agents.

Keywords: *Artemisia campestris*,Polyphenols, Flavonoids,Chlorogenic acid,Antioxidant.

Cezayir'den*Artemisia campestris*'in Hidro-alkolikEkstresininTanımlanması, Nicelik ve AntioksidanAktivitesi

ÖZET

Bu çalışmada, Cezayir*Artemisia campestris*'in hidro-alkolikekstresi, HPLC-DAD-ESI-MS / MS kullanılarak fenolik bileşenleriaçısındanaraştırılmıştır. Ayrıca, in vitro antioksidanaktivite ve toplam fenolik içeriksırasıyla ORAC ve Folin Ciocalteu testleriiledeğerlendirilmiştir.HPLC-DAD-ESI-MS / MS analizi, esasolaraksaptanananabileşiklerin, caffeoylquinic asitizomerleri, flavonoidler ve benzoikasittürevleriolduğunuortayaçıkarmıştır.Ekolarak, hidro-alkolikekstre, 120.5 ± 10.4 µmol TEAC/g DW'ninümitvaatedenantioksidanaktivitedeğerigösterdi ve buaktiviteile 102.09 ± 1.65mg / g GAEDW'nintoplam fenolik içerikdeğeriarasındagüçlübirkorelasyon saptanmıştır

var. Sonuçolarak, *Artemisia campestris*'in hidro-alkolikekstre, doğalolarakoluşanantioksidanajanlarınüretimiiçinumutvericibiradaydır.
AnahtarKelimeler: *Artemisia campestris*, Polifenoller, Flavonoidler, Klorojenikasit, Antioksidan.

INTRODUCTION

The genus *Artemisia* is one of the largest and mostwidely distributed genus of the family*Asteraceae* in Europe and North Africa, which have been characterizedfor their pronounced biological activities and are considered to produce most medicinally important secondary metabolites. Eleven species of *Artemisia* can be found in Algerian flora [1-2].*Artemisiacampestris* 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, anticancer, against digestive troubles, gastric ulcer, and menstrual pain [3-5].*A. campestris* extract was reported to be a potent free radical scavenger of 1,1-diphenyl-2-picryl hydrazyl (DPPH), 2,2'-azinobis3-ethylbenzthiazoline-6-sulfonic *acid* ($ABTS^{\bullet+}$) and superoxide anion radicals ($O_2^{\bullet-}$) 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 [5-8].

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 (3‑CQA). Other isomers are derivative chlorogenic acid 4-*O*-Caffeoylquinic acid (4‑CQA) and 5-*O*-Caffeoylquinic acid (5‑CQA). 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 *Artemisia campestris*usingHPLC-DAD (High-Performance Liquid Chromatographic/Diode Array Detector) coupled with ESI-MS (Electrospray Ionization/Mass Spectrometry). In addition, HPLC-DAD-EC (ElectrochemicalDetector) quantification of phenolic, flavonoid contents and

hydroxycinnamic acid was carried out.Finally, the antioxidant capacity of the extract was also evaluated using 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 UV System equipment (Molsheim, France).

Plant Material

Aerial parts of *A. campestris* were collected fromthe Laghouat region in the northern Algerian Sahara in summer 2015.The identification and authentication of the plant were carried outby Dr. Mohamed Kouidri, 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 under the 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 repeated in triplicate.

Equipments and conditions of analysis

Liquid chromatography with diode array, and electrochemical detection

The High Performance Liquid Chromatography (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 x 4 mm, particle size 5 µm, Merck). The electrochemical detector (ED) Dionex® performed signal measurements by integratedvoltammetry at potentialsbetween -1.0 v and 1.0 v with a scan time of 1.00s. The obtained results were acquired at a frequency of 50Hz using an analog/digital converter. Photodiode array detector was programmed for scanning between 192 and 798nm at a speed of 1Hz with a bandwidth of 5 nm. The detection was monitored using three

individualchannels, 280, 320 and 360 nm, at a speed of 10Hz 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® Water95% (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 a Waters® Alliance 2695 HPLC equipment fitted with a diode array detector (DAD), Waters 2996 (PDA), and a triple quadrupole spectrometer (TQ) (Micromass® Quattro microTM, Waters) with an ESI source operating in negative mode. The capillary in the ESI source was placed at 3.0KV and the cone at 30V. The chromatographic separation was performed on a LiChroCART RP-18 column (250 x 4 mm, particularly from size 5 um, Merck) at 35 ° C. The eluents used were A: formic acid (0.5% v / v) and B: acetonitrile (LC-MS grade). Gradient elution program is applied for chromatographic analysis (Table 1). Flow rate was maintained at 0.3 mL/min and the injection volume was 10 µL. Nitrogen (N_2) ultrapure was used as nebulizer and drying gas and gas. Argon (Ar) ultrapure 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 ofPhenolic 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 performed and final results were expressed in terms of gallic acid equivalents per gram of dry weight (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 performed and final results were expressed in terms of rutin equivalents per gram of dry weight (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 performed and final results were expressed in terms of caffeic acid equivalents per gram of dry weight(mg/g CA DW).Additionally, the content of total phenols was determinedcolorimetrically with Folin'sreagent according to the method ofStamatakis[11]. The phenolic contents were expressed as mg of gallic acid equivalents per gram of dry weight (mg/g GAE DW).

Oxygen radical absorbance capacity (ORAC)

Peroxyl radical scavengingcapacity was determined by the ORAC method. The assay was carried out by followingmethod of Huang [12] modified for the FL800 microplate reader(BioTekInstruments, Winooski, VT, USA) as described by Feliciano[13]. All data were expressed as micromoles of Trolox equivalent antioxidant capacity per gramdry weight. (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 ofthe majority of the compounds.Chromatograms at 280 nm are widely used to study phenolic compounds because absorption at thiswavelengthissuitable to detect a large number of such compounds. The maximum absorption wavelengths (λ_{max}), 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_t) 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_{max} 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 also detected [14].The chromatogram of the hydroalcoholic extract of the aerial parts from *A. campestris* is presented in Fig.1. The most relevant components were caffeoylquinic acids.In general, in the MS^1spectrum 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.

Quantification of chlorogenic acid derivatives of *A. campestris*

The content of chlorogenic acids derivatives of *A. campestris* extract was determined. The amount of the identified compounds is given in Table 2. The 3,4-Dicaffeoylquinic acidwas the major caffeoylquinic acid in the hydroalcolicextract of *A. campestris* (274.76 ± 9.50 mg eq Trolox/L).

The data in Table 2 reveal that the highest quantities of the three isomers of the caffeoylquinic acid (3-*O*-Caffeoylquinic acid191.92±5.4 mg eq Trolox/L), (4,5-Dicaffeoylquinic acid 117.61 ± 3.52 mg eq Trolox/L), and(5-*O*-Caffeoylquinic acid 6.48 ± 0.25 mg eq Trolox/L).

Antioxidant activity and total phenolic content

The antioxidant and total phenolic content of the *A. campestris* extract were measured using ORAC assay, and the result is shown inTable 4.

Discussion

Characterization of Caffeoylquinic Acids (M= 354) and Dicaffeoylquinic acids (M= 516)

Two peaks were detected at m/z 353 and assigned using the hierarchial keys previously developed [15-18] as well-known Chlorogenic acid (3-*O*-Caffeoylquinic acid) and 5-*O*-Caffeoylquinic acid. Two dicaffeoylquinic acid isomers were identified by their parent ion *m/z* 515 and were assigned as 3,4-Dicaffeoylquinic acid and 4,5-Dicaffeoylquinic acid [8,18].

Characterization of other nucleus

A peak was detected at R_t = 27.5 min with $[M-H]^-$ at *m/z* 153 with a characteristic MS^2 fragment at *m/z* 109 $[M-H-44]^-$ due to lose of CO_2 moiety; it was identified as 3,4-dihydroxybenzoic acid (Protocatechuic acid) [19]. Another peak at R_t = 31. 6 min showed a deprotonated molecule $[M-H]^-$ at (*m/z*) 205 with MS^2 fragments of 143, 129 and 114; it was assigned to Quinic acid methyl ester[20]. A molecular ion at R_t = 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_2 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 [21].A peak at R_t = 58.6 showed a deprotonated ion $[M-H]^-$ at *m/z* 367 and MS^n ions at *m/z* 191 equivalent to quinic acid moiety, and another fragment at m/z 173 due to loss of H_2O molecule; it was identified as 4-*O*-feruloylquinic acid [22].Also, a peak at R_t = 73.3 showed a deprotonated ion $[M-H]^-$ at *m/z* 463 and MS^n 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 were appeared at *m/z* 179 and 151; it was identified as Quercetin-*O*-glucoside[23]. A peak at R_t = 76.8 showed a deprotonated ion $[M-H]^-$ at *m/z* 609 and a characteristic MS^n 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)[24].Finally, a peak at R_t = 114.0 showed a deprotonated ion $[M-H]^-$ at *m/z* 313 and a characteristic MS^n ions at *m/z* 298 due to the loss of methyl moiety

[M-H-CH_3]$^-$and 283 due to further loss of another methyl moiety [M-H-2CH_3]$^-$; it was identified as 4′,7′ -dimethoxy luteolin[25].

Antioxidant activity and total phenoliccontent

In the current study, theORAC (Trolox equivalents, TE) value (120.5 ± 10.4μmol TEAC/g DW) is below the results (263.65± 39.7 μmol TEAC/g DW) found by Bakchicheet al.[7], and also stronger than the values of different Artemisia species harvested in Korea that wasreported by Lee et al.[26]. This can be explained for several reasons as the method of extraction, the date and place of the harvest(seasonal variations).

The reagent Folin Ciocalteu is almost used in the quantification of total phenols, this reagent is not specific only for phenols but also it has the ability to reacts with sugar, protein..., ect.So,for this reasonour result is very high. We found a value greater than the values of the total phenols with the same species Djeridaneet al.[27] (20.38 mg / gGAE DW), Bakchiche et al.[7](53.84 mg / g GAEDW).

Bakchicheet al.[7]previously stated that the hydroalcoholic extract from aerial parts of *A. campestris* was shown to possess 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 (161.92 ± 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 that demonstrated the high antioxidant activity and phenolic content was further analysed for the presence of a number of mono(3-*O*-caffeoylquinic, 5-*O*-caffeoylquinicacids) and di (3,4-Dicaffeoylquinic acid, 4,5-Dicaffeoylquinic acid) substituted chlorogenic acid derivatives using HPLC-MS/MS.

Conclusion

In conclusion, 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, flavonoid contents and hydroxycinnamic acid was carried out, and the antioxidant capacity of the extract was also evaluated using ORAC assay.According to the data obtained, 11 phenolic compounds in the hydroalcoholic extract ofwere tentatively identified using HPLC-DAD-ESI-MS/MS technique. The identified compoundscontained 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, *A. campestris*aerial parts are considered a promising source of naturallyoccurring

antioxidant agents, andpolyphenol profile may be regarded as a model for caffeoylquinic acid distribution in the plant *A. campestris* and can help to distinguish the chlorogenic acid isomers.

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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

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

Peak No.	R_t(min)	UV	$[M-H]^-$ *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]

Table 3. Quantification of chlorogenic acids derivatives with electrochemicaldetector.

Peak No.	R_t	*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	274.76± 9.50
10	89.18	515	4,5-Dicaffeoylquinic acid	117.61± 3.52

Table 4. Phenolic,hydroxycinnamic acid and flavonoids contents and value of ORAC assay of *A. 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)
A. campestris extract	61.42 ± 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

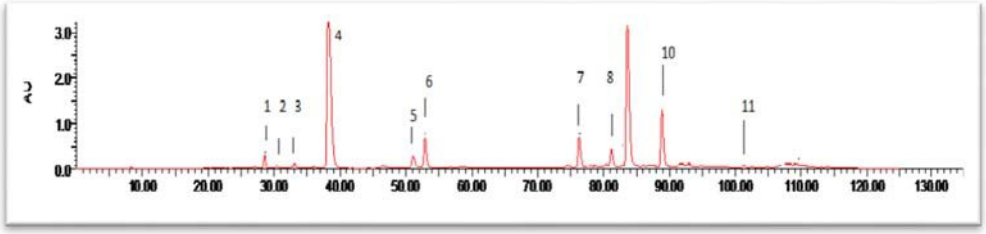

Fig. 1. Chromatographic profile of *A. campestris* obtained by HPLC-DAD at 280 nm.