RESEARCH ARTICLE

Phenolic Profile and Antioxidant Activity of *Centaurea choulettiana* Pomel (Asteraceae) Extracts

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Abstract: ***Aim and objective*:** This study aimed to quantify phenolic compounds in ethyl acetate and *n*-butanol extract of *Centaurea choulettiana* Pomel (Asteraceae) leaves and flowers; compare the antioxidant activity of their extracts, identification and quantification of their phenolic acids.

***Materials and methods*:** Both organs extracts of *Centaurea choulettiana* Pomel were investigated and evaluated for their potential antioxidant properties using total phenolics and flavonoids content, DPPH radical scavenging and lipid peroxidation inhibition assays. HPLC-TOF/MS analyses were carried out to identify and quantify some phenolic acids.

***Results*:** The amounts of phenolic and flavonoid content were higher in ethyl acetate extract of leaves (325.81 ± 0.038 mgGAE and 263.73 ± 0.004 mgQE /g of extract) respectively. Besides, this extract exhibited the most powerful effect on the DPPH radical scavenging activity with (96.54%), on lipid peroxydation inhibition (64.17%). Ethyl acetate extract of leaves and flowers were found to contain almost the same phenolic compounds, with the leaves having the highest values. Chlorogenic acid was detected in the *n*-butanol extract of flowers with the highest concentration 17.78 mg/kg plant.

***Conclusion*:** The ethyl acetate extract of leaves of *Centaurea choulettiana* possesses strong antioxidative properties *in vitro*. They are confirmed by high polyphenols and flavonoids content. The HPLC-TOF/MS analysis reveals the presence of 4-hydroxybenzoic acid, gentisic acid, chlorogenic acid, caffeic acid, vanillic acid, *p*-Coumaric acid, ferulic acid, salicylic acid and protocatechuic acid.

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INTRODUCTION

Plants are a precious source of interesting biologically active compounds. Biological and chemical screenings are complementary approaches for the rapid detection of plant constituents. Phenolic compounds are a wide group of secondary metabolites that can be found in plants, such as simple phenols, phenolic acids, flavonoids, coumarins, tannins, etc [1]. Polyphenols which are considered as one of the important phytochemicals having antioxidant properties and several industrial applications gained special interest in the last decades.

The genus *Centaurea* which belonging to the Asteraceae family contains about 700 species essentially centered in the Mediterranean region [2]. In Algeria, it is represented by 45 species including 7 in the Sahara [3-5]. However, many species of this genus have long been used in traditional medicine for cure of various ailments such as antidiabetics [6], diuretic [7] and antirheumatic [8], as well as for the treatment of cancer [9] and microbial infections [10]. A variety of secondary metabolites have been reported from different species of this genus such as sesquiterpene lactones and flavonoids [11-16] which have been shown to be biologically active [17-19]. *Centaurea choulettiana* Pomel is a perennial herb, from the Asteraceae family. This plant is often synonymous with *Centaurea acaulis* L. ssp. *Balansae* Boiss. et Reut. [3]. As a part of our continuing investigation of Algerian medicinal plants [11-14, 20-23], we report in the present study, the identification and quantification of phenolic acids in EtOAc and *n*-BuOH extracts of CcL and CcF of *C. choulettiana* using HPLC-TOF/MS. The later

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extracts were subjected to DPPH radical scavenging activity and lipid peroxidation inhibition tests to evaluate the antioxidant activities. To our knowledge, no report on any phytochemical or pharmacological study on *C. choulettiana* is available up to date.

MATERIALS AND METHODS

Plant Material

The plant material was collected from the east of Algeria (M'Sila region) in June 2011 and authenticated by Dr. D. Sarri (University of M'Sila). A voucher specimen (ACC06/11) has been deposited in the Herbarium of the VARENBIOMOL Unit Research, University Frères Mentouri Constantine, Algeria.

Extraction Procedure

A quantity of 1370 g (leaves) and 840 g (flowers) were dried at room temperature and cut into small pieces then macerated three times (24h for each time) with methanol / H_2O (70%). After filtration and evaporation, the obtained extract was partitioned with solvents in increasing polarity: chloroform, ethyl acetate and *n*-butanol. Each extract was evaporated under reduced pressure. The obtained extracts were (2.37 g leaves and 1 g flowers) of $CHCl_3$, (3 g leaves and 2 g flowers) of EtOAc and (14 g leaves and 10 g flowers) of *n*-BuOH respectively.

Preparation of Calibration Curves

The standard solutions (gallic acid, gentisic acid, chlorogenic acid, 4-hydroxybenzoic acid, protocatechuic acid, caffeic acid, vanillic acid, 4-hydoxybenzaldehyde, *p*-coumaric acid, ferulic acid, rosmarinic acid, salicylic acid) were freshly prepared by dissolving authentic compounds in methanol. The required concentration standard solutions were made by gradual dilution with methanol. The calibration curve was constructed for each standard by plotting the concentration of standard against peak area.

Chromatographic Conditions

The HPLC analysis was performed with an Agilent Technology of 1260 Infinity HPLC System equipped with 6210 Time of Flight (TOF) LC/MS detector and ZORBAX SB-C18 (4.6 x 100 mm, 3.5 µm) column. Mobile phases A and B were ultra-pure water with 0.1% formic acid and acetonitrile, respectively. Flow rate was 0.6 ml min^{-1} and column temperature was 35 °C. Injection volume was 10 µl. The solvent program was as follow: 0-1 min 10% B; 1-20 min 50% B; 20-23 min 80% B; 23-30 min 10% B. Ionization mode of HPLC-TOF/MS instrument was negative and operated with a nitrogen gas temperature of 325 °C, nitrogen gas flow of 10.0 L min^{-1}, nebulizer of 40 psi, capillary voltage of 4000 V and finally, fragmentor voltage of 175 V. For sample analysis, standards phenolic acids and dried crude extracts (200 ppm) were dissolved in methanol at room temperature. Samples were filtered passing through a PTFE (0.45 µm) filter by an injector to remove particulates.

Antioxidant Activity

Estimation of Total Phenolic Content

The amount of phenolic compounds in both extracts was determined by Folin-Ciocalteu reagent method with some modifications [24]. 0.5 ml of Folin-Ciocalteu reagent (1N) and 7.5 ml of H_2O, 1.5 ml of 10% solution of Na_2CO_3 were added to 1 ml of plant extract. The resulting mixture was incubated for 90 min. The absorbance of the sample was measured at 765 nm. Gallic acid was used as standard (1 mg/ml). The results were determined from the standard curve and were expressed as Gallic acid equivalent (mg GAE/g of extract).

Estimation of Total Flavonoid Content

1 ml of sample plant extract was mixed with 1 ml of methanol, 5 ml of H_2O, 0.3 ml of $NaNO_2$ (5%), then 0.6 ml of $AlCl_3$ (10%) was added and remains at room temperature for 5 min. The absorbance was measured at 510 nm; using quercetin as standard (1 mg ml^{-1}). Flavonoid contents were determined from the standard curve and were expressed as quercetin equivalent (mg QE/g of extract) [24].

DPPH Scavenging Activity

The antioxidant activity was evaluated by monitoring its ability in quenching the stable free radical DPPH [25]. Six different methanol dilutions of each crude extracts were mixed with 1 ml of DPPH 0.2 mM methanol solution. After 30 min, the readings were made at 517 nm. A solution of DPPH (1 ml, 0.2 mM) in methanol (1 ml) was used as a negative control and ascorbic acid in the same concentrations used for the fractions and the crude extracts provide the positive control. The test was performed in triplicate. The percentage of DPPH scavenging activity (I%) was calculated using the following equation (1):

$$\mathbf{I\% = (A_0 - A_1 / A_0) \times 100}.$$

Where $\mathbf{A_0}$ is the absorbance of the control

$\mathbf{A_1}$ is the absorbance of the extract or standard.

Inhibition of Lipid Peroxidation

Inhibition of lipid peroxidation activities of EtOAc and *n*-BuOH extracts were estimated by the method of thiocyanate by using linoleic acid as a source of peroxide [26]. The generated peroxide reacted with the ferrous chloride (Fe^{2+}) to form the ferric ion (Fe^{3+}) monitor form of a thiocyanate complex. The emulsion of linoleic acid was prepared by homogenization of linoleic with Tween-40 emulsified in phosphate buffer (0.2 M, pH 7). The samples were prepared in MeOH/water and the selected concentrations for the study were 800 and 1000 µg ml^{-1}. After incubation at 37 °C the ammonium thiocyanate and ferrous chloride were added. The mixture prepared by the same procedure was used as control. Trolox was used as reference. The coloration was measured at 500 nm for 4 successive days; the results were calculated according to (1):

Statistical Analysis

All assays were carried in triplicates and results expressed as means ± standard deviation. Statistical comparisons were done with the Student's test. Differences were considered to be highly significant at $P< 0.01$ and significant at $P< 0.05$.

RESULTS AND DISCUSSION

Identification and Quantification of Phenolic Acids by HPLC-TOF/MS

The EtOAc as well as *n*-BuOH extracts of CcL and CcF of *Centaurea choulettiana* were analyzed by HPLC-TOF/MS. Table **1** presents the amounts of individual phenolic acids, of which the identification has been performed on the basis of their retention times and mass spectrometry by comparison with those of different standards.

The separation of phenolic acids in EtOAC and *n*-BuOH extracts of CcL and CcF is shown by chromatograms represented in Fig. **1**. These phenolic acids were identified as gentisic acid, chlorogenic acid, 4-hydroxybenzoic acid, caffeic acid, vanillic acid, *p*-coumaric acid, ferulic acid, salicylic acid and protocatechuic acid. On one hand the EtOAc extracts of CcL and CcF were found to contain almost the same phenolic compounds, with the leaves having the highest values. The *n*-BuOH extract of CcL was found to contain all of the previous compounds with caffeic acid, chlorogenic acid and ferulic acid having the highest concentrations (10.07, 5.05, 4.81 mg/kg plant) respectively.

On the other hand, the same previously mentioned phenolic compounds were detected in the EtOAc extract of CcL with 4-hydroxybenzoic acid and gentisic acid having the highest concentrations (5.43, 3.24 mg/kg plant) respectively. Finally, chlorogenic acid was detected in the *n*-BuOH extract of CcF with the highest concentration 17.78 mg/kg plant.

Evaluation of the Antioxidant Activity

Determination of Total Phenolic and Flavonoid Contents

The EtOAc extracts contain more phenolic and flavonoid compounds than *n*-BuOH extracts. However, the leaves extracts apparently contain more phenolic and flavonoid compounds than the flowers. The results obtained in the present study as shown in Table **2**, revealed that the leaves of both EtOAc and *n*-BuOH extracts had the highest amount of phenolic content (325.81 ± 0.038 mg GAE and 176.91 ± 0.003 mg GAE/g of extract) respectively. Among the different extracts tested for total flavonoid content, EtOAc and *n*-BuOH extracts of CcL reflected the highest amount (263.73 ± 0.004 mg QE and 145.2 ± 0.001 mg QE/g of extract) respectively.

DPPH Radical-Scavenging Activity Assay

The free radical scavenging activities of the four extracts were evaluated through their ability to quench 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical.

Comparing the four extracts, the EtOAc of CcL impact was more effective than the others, and its percentage of the inhibition was 94.29% at 200 µg/ml. The *n*-BuOH extract of CcF exhibited the weakest effect (55.3%).

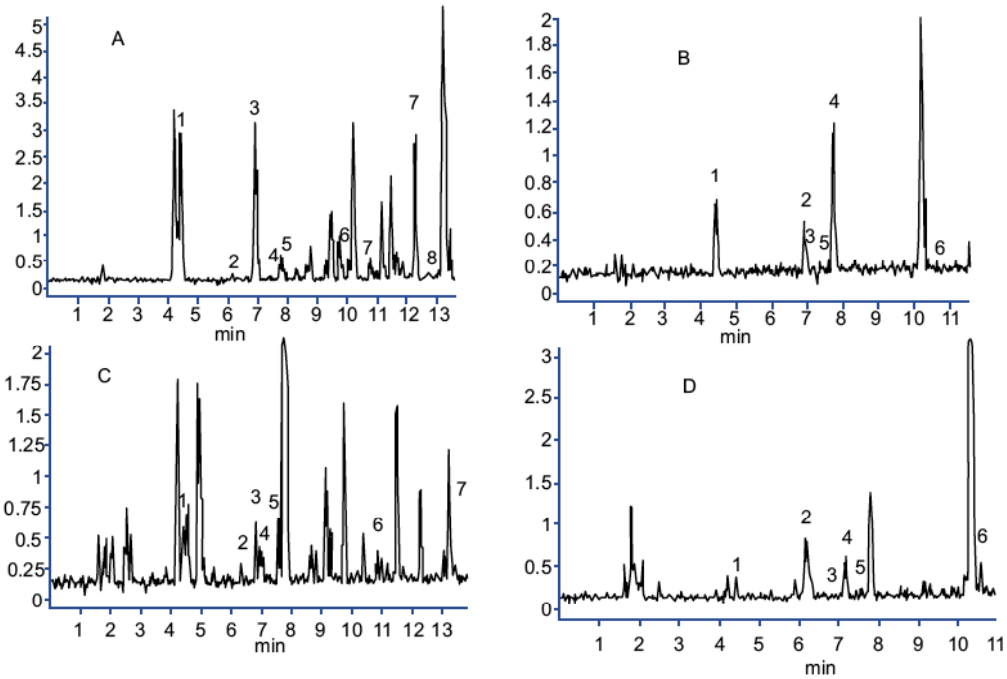

Fig. (1). HPLC-TOF/MS chromatograms of phenolic acids in extracts of *Centaurea choulettiana* pomel: 1.gentisic acid, 2.chlorogenic acid, 3. 4-hydroxybenzoic acid, 4.caffeic acid, 5.vanilic acid, 6.*p*-coumaric acid, 7.ferulic acid, 8.salicylic acid.
A: ETOAc (leaves) extract, B: ETOAc (flowers) extract,
C: *n*-BuOH (leaves) extract, D: *n*-BuOH (flowers) extract.

Table 1. HPLC-TOF/MS analysis of phenolic acids from *Centaurea choulettiana* extracts.

Phenolic acids	RT	EtOAc leaves	EtOAc flowers	*n*-BuOH leaves	*n*-BuOH flowers
gentisic acid	4,40	3.23	0.79	3.01	1.70
chlorogenic acid	6,15	1.13	ND	5.04	17.78
4-hydroxybenzoic acid	6,90	5.43	0.82	2.91	0.21
protocatechuic acid	7,00	ND	0.22	2.68	1.25
caffeic acid	7,71	0.10	0.49	10.07	0.11
vanilic acid	7,82	0.41	0.12	0	ND
p-coumaric acid	10,01	0.28	0	0	0
ferulik acid	10,82	0.50	0.55	4.81	0.04
salisilic acid	13,04	0.09	0	0.14	0

ND: not detected

Table 2. Total phenolic and flavonoid values of *Centaurea choulettiana* Pomel.

Extracts	polyphenols	flavonoids
EtOAc (leaves)	325.81 ±0.038	263.73±0.004
EtOAc (flowers)	248.35±0.005	188.20±0.013
***n*-BuOH (leaves)**	176.91±0.003	145.20±0.001
***n*-BuOH (flowers)**	133.13±0.002	122.33±0.003

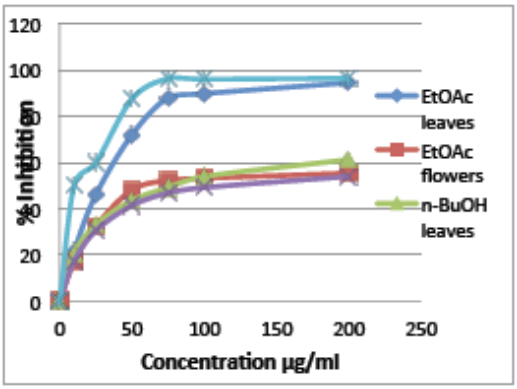

Fig. (2). DPPH scavenging activities of *Centaurea choulettiana* Pomel extracts.

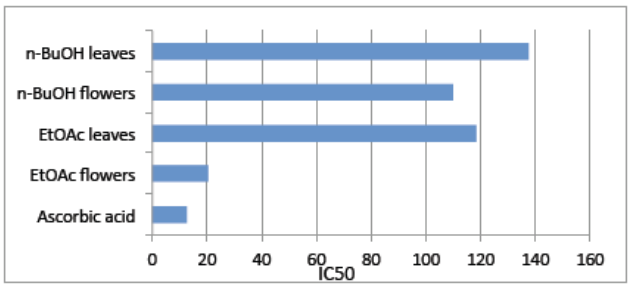

Fig. (3). DPPH IC50 values of antioxidant activity of *Centaurea choulettiana* Pomel extracts and Ascorbic Acid.

Inhibition of Lipid Peroxidation

Lipid peroxidation represents the main molecular mechanism involved in the oxidative damage of cell structures leading to cell death observed in several pathologies.

Figure **4** showed the percentage of linoleic acid inhibition of various extracts for two different concentrations (800 and 1000 µg/ml), which was in the following order:

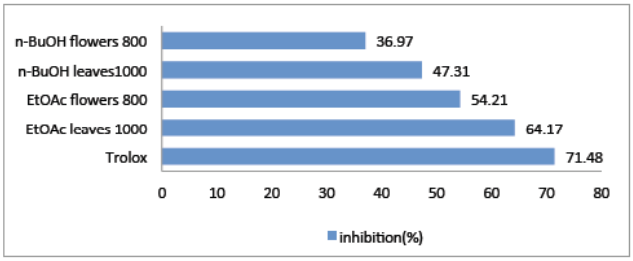

Fig. (4). LPO inhibition (%) *Centaurea choulettiana* Pomel.

Trolox (71.5%), AcOEt (54.2–64.2%) and *n*-BuOH (37–47.3%).

Comparing the results at 1000 µg/ml, it is clear that EtOAc (64.2%) possess better inhibitory effect than *n*-BuOH (47.3%).

Figure **5** showed an increase in absorbance of the control which has reached the maximum (1.6 ± 0.02) at 72 hours. The maximum absorbance of control reflecting the formation of LPO did not exceed (0.44 ± 0.03) at 72 hours. The decrease in absorbance of EtOAc extract could be attributed to the decomposition of linoleic acid hydroperoxide during peroxidation.

According to the nature and total phenolic concentrations obtained in this investigation, it is clear that *Centaurea choulettiana* extracts displayed effective free radical scavenging properties as reflected by the suppression of DPPH radical and lipid peroxidation initiated by linoleic acid emulsion system by ferric thiocyanate. Linoleic acid, as part of fatty acids embedded in cell membrane; was more

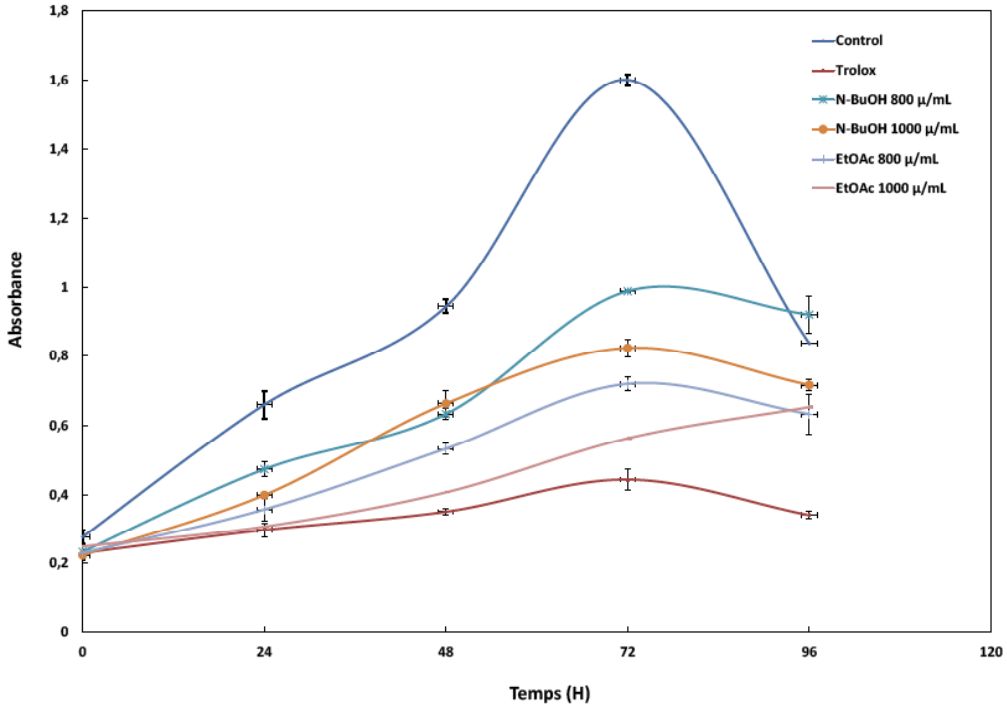

Fig. (5). LPO absorbance of *Centaurea choulettiana* Pomel.

sensitive towards attack by free radicals that cause lipid peroxidation [27]. It has been suggested that the antioxidant activity of plants might be due to their phenolic compounds [28-29]. According to the obtained results, both CcL and CcF EtOAc extracts of *C. choulettiana* showed the highest amount of TPC and were richer in flavonoids (TFC) as well. Flavonoids and phenolic acids are major groups of compounds that act as primary antioxidants or free radical scavengers [30]. Both EtOAc and *n*-BuOH extracts (CcL and CcF) of *C. choulettiana* were found to contain mostly the same polyphenolic compounds. In this investigation, the antioxidant activity of different extracts of *C. choulettiana* could be due to one or more components acting in synergetic manner of the above mentioned chemical constituents. The differences between the extracts can be probably explained by the presence of more active substances in the EtOAc such as 4-hydroxybenzoic acid, gentisic acid, which were the highly concentrated in comparaison to *n*-BuOH extract characterized by the presence of important amounts of caffeic acid, chlorogenic acid, ferulic acid with no direct relation to its antioxidant effects.

CONCLUSION

The results indicate that the EtOAc extract (leaves) of *Centaurea choulettiana* possesses strong antioxidative properties *in vitro*. They are confirmed by high polyphenols and flavonoid contents. And the HPLC-TOF/MS analysis reveals the presence of 4-hydroxybenzoic acid, gentisic acid, chlorogenic acid, caffeic acid, vanillic acid, *p*-coumaric acid, ferulic acid, salicylic acid, protocatechuic acid.

ABBREVIATIONS

EtOAc	=	Ethyl acetate extract
CcL	=	*Centaurea choulettiana* of leaves
CcF	=	*Centaurea choulettiana* of flowers
n-BuOH	=	*n*-butanol extract
HPLC-TOF/MS	=	High Performance Liquid Chromatography-Time of Flight/Mass Spectrometry

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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