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Screening of Antioxidant Potentials and Bioactive Properties of the Extracts Obtained from Two *Centaurea* L. Species (*C. kroumirensis* Coss. and *C. sicula* L. subsp *sicula*)

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Abstract: Herbal medicines, dietary supplements, or infusions with health promoting effects can note strong consumer demand. Hence, researchers and scientists have been increasingly focusing on the promotion of medicinal plants. In this respect, the present study has the evaluation of the biological properties of different extracts (hexane, chloroform, methanol, ultrasonic, essential oil, and supercritical) obtained from two *Centaurea* species (*C. kroumirensis (Coss.)* and *C. sicula* L. subsp *sicula*) as purpose. Antioxidative activities were assessed by in vitro assays including total phenolic content, free radical scavenging assay and ferric ion reducing power. Among the fourteen extracts examined, the highest total phenolic content (TPC) and antioxidant abilities were recorded in ultrasonic ethanol extracts of both *Centaurea* species. Additionally, these extracts exhibited a noticeable strong protective effect of the human skin fibroblast cell line (HS-68) that was exposed to oxidative stress induced by hydrogen peroxide. According to our results, the sonication with ethanol might be an ideal, rapid, and eco-friendly extraction method for obtaining a polyphenol-rich extract that exhibited high antioxidant activity. The results presented herein suggest that *Centaurea* species could be promoted as an antioxidant resource that could be exploited for the development of nutraceuticals or in pharmacology industry.

Keywords: *Centaurea spp*; antioxidant; phenolics; plant extract; protective effect

1. Introduction

Recently, free radicals have been the concern of several researches. These free radicals are naturally produced, in cellular systems, in the form of endogenous reactive from oxygen/nitrogen species (ROS/RNS) through natural metabolic reactions [1]. In addition, several external sources (e.g., pollution, UV radiations, alcohol, smoking, and chemicals) raise oxidative stress, causing an overproduction of free radicals in the human body [2]. Naturally, the endogenous antioxidant defense systems (SOD, catalase, and peroxidase) control their production and reduce the over amount of these compounds.

Other exogenous antioxidant (i.e., vitamin E, vitamin C, and β–carotene) also contribute to scavenge these free radicals. However, in excess production, these antioxidants become inefficient. Free radicals can negatively alter lipids and proteins engendering peroxidation that can cause undesirable alterations of cells and gene mutation [3], premature aging, tissue injury, and inflammation [4]. Ultimately, these free radicals lead to the development of cancer, neurodegenerative diseases, cardiovascular diseases, hypertension, Alzheimer's disease, and diabetes mellitus, [5–7]. Several synthetic dietary antioxidants have been recognized by their ability to prevent or limit the oxidative damage. However, the most commonly used (i.e., butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), tert-butylhydroquinone (TBHQ), and α-tocopherol) have been restricted in the food sector and been claimed to be carcinogenesis and responsible of liver damage [8]. Therefore, researchers are increasingly interested in finding alternative antioxidants, safe, natural, and economical. The challenge was to screen natural, abundant, and low-value raw products, particularly with the aim of developing the sustainability concept. Plants and forest biomass, as well as vegetables and fruits, represent a large source of bioactive molecules, particularly, natural antioxidants [2,9]. Phenolic compounds are recognized as healthy phytochemicals due to their potential redox. They reduce oxidative stress, thereby decreasing the risk of degenerative diseases and inhibiting macromolecular oxidation [10].

Centaurea is one of the most important genus within the family Asteraceae [11]. It presents more than 600 species, distributed particularly in the Mediterranean region and west Asia [12]. Previous phytochemicals reports have indicated that *Centaurea* genus is rich of sesquiterpene lactones [13], flavonoids [14], flavonoid glycosides [15], lignans [16], alkaloids [17], triterpenes [18], and anthocyanins [14]. There is still growing worldwide interest in the antioxidant ability of phenolics of this genus. Several studies have reported that many *Centaurea* species, such as *C. cyanus* L [19], *C. bornmuelleri Hausskn* [20], *C. drabifolia subsp. drabifolia* and *C. lycopifolia* [21], *C. amaena* Boiss. & Balansa and *C. aksoyi* Hamzaoglu & Budak [1], *C. pseudoscabiosa* Boiss et Buhse subsp. *Araratica* (Azn.) Wagenitz, *C. pulcherrima* Willd var. *pulcherrima* Willd, *C. salicifolia* Bieb. ex Willd. subsp. *abbreviata C.* Koch, and *C. babylonica* (L). Aktumsek et al. [12] exhibited remarkable antioxidants properties that allow for their use as a potential source of new nutraceutical products.

The imbalance between the production and elimination of (ROS) and (RNS), leading to an increase in steady-state or transient ROS concentration, with consequences of cellular physiology, has been defined as oxidative stress [22]. Among the main ROS, hydrogen peroxide (H_2O_2) has revealed DNA oxidative injury in cells and lipid peroxidation [23]. Thereby, the ultimate target of the present research was to investigate whether *Centaurea* plant extracts are able to alleviate H_2O_2-caused oxidative damage on the human Fibroblast HS-68 cell line.

No information on biological properties (i.e., antioxidant ability and protective effect against oxidative stress in cells) was reported so far on of aerial parts of *Centaurea kroumirensis (Coss.)* and *Centaurea sicula* L. subsp *sicula* to the best of author's knowledge.

Hence, the current study presents the first report on the in vitro antioxidant properties of these two *Centaurea* species, one of them being endemic to Tunisia flora. The main objectives were to evaluate the total phenolic content, to assess the bioactive activity through in vitro assays and in cells. The effect of different conventional and non-conventional extraction methods on the antioxidant abilities of both *Centaurea* species has been studied in order to make systematic comparison and select the extract with high antioxidant power for further studies. Moreover, correlation between antioxidant capacities and total phenol content and were evaluated.

2. Materials and Methods

2.1. Chemicals

The pure extraction solvents (i.e., Hexane, chloroform, methanol and ethanol) were bought from Sigma Aldrich (Monastir, Tunisia). 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH%), gallic acid (GAE), Folin and Ciocalteu's phenol reagent, iron (III) chloride ($FeCl_3$), human skin

fibroblasts (Sigma® HS-68), Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum, glutamine, penicillin–streptomycin, and N-acetyl cysteine (NAC) were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Plants Material

Two *Centaurea* species (*Centaurea kroumirensis (Coss.)* and *Centaurea sicula* L. subsp *sicula*) were collected from the north western region of Tunisia (Tabarka region, Fernana, Kroumirie Mountains, Tunisia) during the flowering season of 2017. The plants were identified by botanist Ridha El Mokni. Voucher specimen were deposited in herbarium of Laboratory of research of Botany, Cyptogamy, and Plant Biology Department of Pharmaceutical Sciences, Faculty of Pharmacy of Tunisia.

2.3. Preparation of the Extracts

2.3.1. Hydrodistillation Procedure (HD)

One hundred grams of grounded dried plant were submitted to extraction with hydrodistillation while using Clevenger apparatus, in accordance with the British Pharmacopoeia (1980), with 1 L of distilled water for 5 h (until no more essential oil was extracted). The essential oil was recovered, dried under anhydrous Na_2SO_4, and then refrigerated at 4 °C until further analysis.

2.3.2. Conventional Extraction with Soxhlet Apparatus (SE)

The dried aerial plant materials were ground using Cam international laboratory grinder (Igea Marina, Italy). Forty grams of powdered plant were placed in a thimble-holder and gradually filled with 400 mL of condensed fresh solvent from a distillation flask. The extraction was performed by increasing the solvent polarity (hexane-chloroform-methanol) for 6 h for each solvent. The extracts were concentrated under reduced pressure while using the rotary evaporator (Hei-vap Presición HL G3 from Heidolph technologies) at 40 °C. The prepared extracts were stored at 4 °C in dark until further handling.

2.3.3. Ultrasound-Assisted Extraction (UAE)

The sonication was performed in ultrasonic homogenizer (UIP1000hd from Hielscher Technologies). Ten grams of powdered plant were mixed with 100 mL of absolute ethanol and extraction was carried out for 10 min. at 750 W and 5 kHz. The temperature was regulated and maintained at 25 °C with water circulating from a thermostat bath by means of a pump. The ultrasonic extracts (UE) were centrifuged for 10 min. at 4000 rpm. The supernatants were removed and then evaporated under vacuum by using a rotary vacuum evaporator. The aliquots were then weighed and kept refrigerated at 4 °C in darkness prior to further analysis.

2.3.4. Supercritical Fluid Extraction (SFE-CO_2)

The non-conventional extraction was based on supercritical carbon dioxide extraction with 99.9% CO_2 in a pilot-plant-scale supercritical fluid extractor (Aerospace Technology, Zunyi, China, model SUS304) comprising two-cylinder extraction cells (1 L and 5 L) and two different separators (S1 and S2), each with independent control of temperature and pressure. The extraction system also includes a recirculation pump to condense CO_2. The Cell extractor (50 mm inner diameter and 250 mm length) was filled with 250 g of dried ground plant. Two different supercritical extraction conditions (i.e., the working temperature and pressure) using neat CO_2 were tested. The first extraction was carried out at P = 10 MPa, T = 40 °C (SFE-I), in the second extraction (SFE-II) the pressure and temperature have been increased to at 40 MPa bar and 60 °C, respectively. In both cases, the extraction was performed during 30 min. and CO_2 flow rate was maintained as constant (20 $L{\cdot}h^{-1}$). The supercritical extracts were collected in amber vials, weighed, and then stored at 4 °C.

2.4. Total Phenolic Content

The determination of the total polyphenol content (TPC) in *Centaurea* extracts was performed according to the Folin–Ciolcateau method [24,25]. An amount of 25 µL of each extract solution (4000 µg/mL) were mixed with 12.5 µL Folin–Ciocalteu reagent (diluted 1:1 with ethanol) and 150 µL ultrapure distilled water. The volumetric flasks were vigorously shaken in darkness at room temperature. After 5 min., 25 µL of Na_2CO_3 (2%) solution was added and the mixtures were again incubated for 1 h by intermittent shaking in the dark, at room temperature. The absorbance was measured at 725 nm by the means of an UV/V is spectrophotometer (Varian Cary 50 Scan, Palo Alto, CA, USA). TPC was quantified from the average of three independent experiments with reference to a standard curve of gallic acid and the results were expressed as gallic acid equivalents (GAE) per g of the *Centaurea* dw [26].

2.5. Antioxidant Properties

2.5.1. DPPH Scavenging Activity

The free radical scavenging ability of *Centaurea* extracts was determined while using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay according to the previous work of Messina et al. [25]. The dried samples were re-suspended in ethanol and analyzed at various concentrations (from 0.125 to 4 mg/mL). Forty microliter of each extract was mixed with 160 µL of DPPH ethanolic solution (0.1 mM). The mixture was vigorously shaken and incubated in darkness for 30 min. Absorbance was recorded at 517 nm. Each sample was measured in triplicate. The results were reported as IC_{50} ± SD (i.e., Inhibitory Concentration to cause a 50% decrease of the absorbance). A lower IC_{50} value indicates higher antiradical activity.

2.5.2. Reducing Power Activity (RP)

The reducing power of *Centaurea* extracts was determined according to the spectrophotometric detection of Fe(III)–Fe(II) reduction method involving gallic acid as standard [25,27]. Aliquots of solutions at different concentrations (300 µL) were mixed with 300 µL of phosphate buffer (0.2 M, pH 6.6) and 300 µL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%), the mixture was allowed to stand for 20 min. at 50 °C. After the incubation, 300 µL of trichloroacetic acid (1%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Distilled water (50 µL) and $FeCl_3$ (100 µL, 0.1%) was mixed with 50 µL of the upper layer of solution. Absorbance was measured at 700 nm. The EC_{50} value (i.e., the effective concentration of the extract at which the absorbance was 0.5) was calculated from linear regression analysis.

2.6. Cell Culture

The human HS-68 skin fibroblast cell lines were cultured in suspension in Dulbecco's Modified Egale's Medium (DMEM) supplemented with 10% (*v*/*v*) inactivated fetal bovine serum, 100 µg/mL penicillin-streptomycin, and 2 mM glutamine and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cell culture was carried out under sterile conditions using a grade (II) flow hood.

2.7. Protective Effect, against Induced Oxidative Stress, in Human Skin Fibroblast (HS-68) Cells

The cytotoxic effect of *Centaurea* extracts was tested in the human fibroblast cell (HS-68) using MTT assay. This test was based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to a colored formazan product by mitochondrial dehydrogenase [28]. The rested doses of *Centaurea* ethanolic extracts were determined based on previous studies [26,29,30]. The tested extracts were reconstituted with ethanol as stock solutions and then diluted with fresh medium at five different concentrations in the range of [0.02–0.32] µg GAE/mL. The final concentration of ethanol in treated cells was less than 0.1% (*v*/*v*). The confluent cells were trypsinized and seeded at a density of

7×10^3 cells/well in a 96-well microculture plate and then incubated for 24 h. Preliminary tests have been conducted to evaluate the effects of dose of *Centaurea* extracts on vitality on fibroblast cells to individuate the adequate range of compound concentrations for performing oxidative stress induction experiments. Different doses of Ethanol (ultrasonic) (UE) were added to cells previously inoculated on a 96-well plate. Cell viability was assessed after incubation for 24 h. After the individuation of the concentration that does not induce significant cell mortality, 0.16 µg/mL was selected for both *C. kroumirensis* and *C. sicula*. Subsequently, the cells were exposed to hydrogen peroxide (HP) (50 µM), chemical promoter of oxidative stress, and allowed to incubate for 2 h at 37 °C, according to [25]. Additional wells were treated with the synthetic antioxidant N-acetyl cysteine NAC (10 µM) and gallic acid (GAE) to the same concentration, another set of samples were not treated with *C. kroumirensis* and *C. sicula* extracts, but only with the inducer of oxidative stress (HP) Each sample was analyzed in quintuplicate. The percentage of viable cells has been determined with respect to the control values and expressed as the mean ± SD.

2.8. Statistical Analysis

All of the measurements were performed in triplicate and the results were expressed as average ± SD. Analysis of variance (ANOVA) was performed to investigate statistical evaluation for each sample. The differences among the mean values were assessed using the Student–Newman–Keuls or Games Howell test, depending on the homogeneity of the variables test. The homogeneity of variance was confirmed by the Levene test and a 95% level ($p < 0.05$) was considered to be significant. The Pearson correlations have been evaluated. All of the data were analyzed by SPSS ® software (version 20.0, SPSS Inc., Chicago, IL, USA).

3. Results and Discussions

3.1. Total Phenolic Content (TPC)

Polyphenols are among the major secondary metabolites in medicinal and dietary plants known to possess important biological activities particularly antioxidant abilities [12]. Therefore, the assessment of TPC in the studied extracts is very important. Table 1 summarizes the TPC in the *Centaurea* extracts.

Table 1. Total phenolic, 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) radical scavenging activities and ferric reducing power of *Centaurea kroumirensis* (Coss.) and *Centaurea sicula* L. subsp *sicula* extracts. Values are expressed as means ± SD ($n = 3$). Analysis of variance (ONE-way ANOVA) was used for comparisons.

Plants	Extracts	TPC (mg GAE/g)	DPPH IC50 (mg/mL)	RP EC50 (mg/mL)
Centaurea kroumirensis (Coss.)	Hexane (H)	18.34a ± 0.31	0.49a ± 0.01	6.28a ± 0.77
	Chlorofom (C)	43.06b ± 0.16	0.58a ± 0.02	12.36b ± 0.41
	Methanol (M)	61.12c ± 0.60	0.59a ± 0.01	8.77c ± 0.16
	Ethanol (ultrasonic) (UE)	42.73b ± 0.36	0.94a ± 0.01	5.15a ± 0.09
	supercritical (I) (SFE(I))	4.79d ± 0.15	30.93b ± 1.24	65.17d ± 2.93
	supercritical (II) (SFE(II))	13.17e ± 0.05	21.22c ± 0.50	22.44e ± 1.12
	essential oil (EO)	12.64e ± 0.20	45.10c ± 0.27	24.32e ± 0.46
Centaurea sicula L. subsp sicula	Hexane (H)	8.32a ± 0.50	25.20a ± 1.55	21.50a ± 0.33
	Chlorofom (C)	28.25b ± 0.72	1.96b ± 0.05	7.62b ± 0.15
	Methanol (M)	23.27c ± 0.077	2.94b ± 0.02	20.55a ± 0.76
	Ethanol (ultrasonic) (UE)	24.56d ± 0.05	2.00b ± 0.10	11.75c ± 0.58
	supercritical (I) (SFE(I))	8.75a ± 0.87	28.01c ± 1.08	22.98d ± 0.59
	supercritical (II) (SFE(II))	12.29e ± 0.21	22.25d ± 0.62	24.30e ± 0.90
	essential oil (EO)	9.28f ± 0.27	28.36d ± 2.01	25.05f ± 0.68

Means in the same column followed by different letters at superscript are significantly different ($p < 0.05$).

The values ranged from 61.11 to 4.79 mg GAE/g and from 28.25 to 8.32 mg GAE/g for *C. kroumirensis* and *C. sicula*, respectively. Polar methanol (M), ethanol (ultrasonic) (UE), and moderately polar chlorofom (C) extracts presented the highest concentration of polyphenolics for both studied plants, as shown in Figure 1.

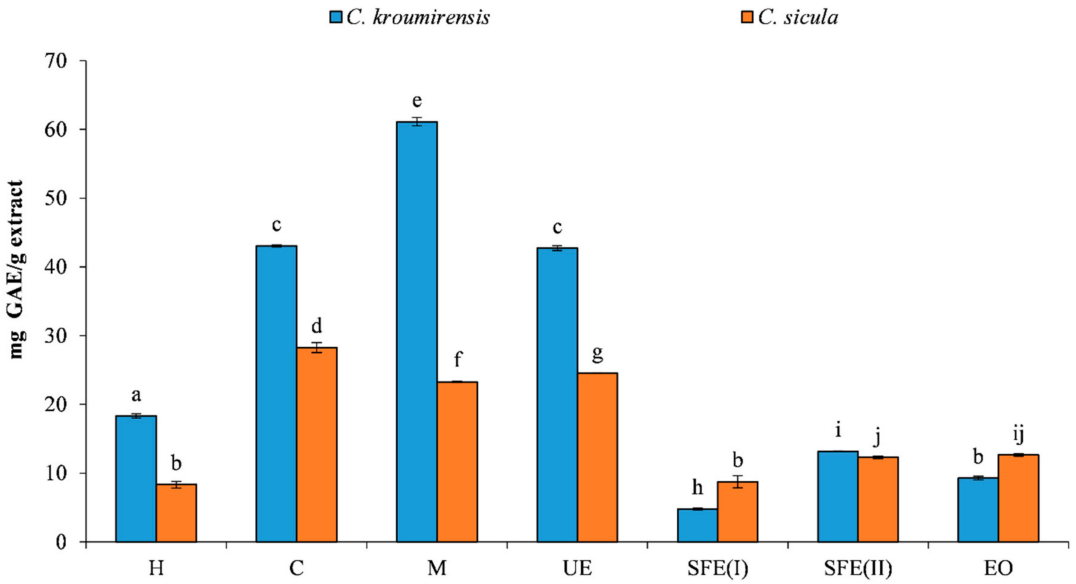

Figure 1. Total phenolic content (mg GAE/g) in extracts obtained from aerial part of two *Centaurea* species (*C. kroumerienis* and *C. sicula*) with different extraction techniques (Different letters indicate significant difference ($p < 0.05$)).

According to the species (Figure 1), the highest polyphenol contents was recorded in *C. kroumirensis*. This plant exhibited 61.117 ± 0.599 mg GAE/g in M extract. The C and UE extracts contained similar levels of polyphenolic contents (43.06 ± 0.158 and 42.729 ± 0.357 mg GAE/g, respectively), which point out that solvent and extraction techniques greatly influence the polyphenolic content of the plant extracts. These findings are in agreement with the previous studies, which reported higher concentrations in polyphenolics in the extract that was obtained with methanol and ethanol [21,31,32]. However, the essential oil (EO) and supercritical SFE(I-II) extracts that were obtained without co-solvent had the lowest TPC for both *Centaurea* species. The obtained results are predictable, since there is a positive correlation between the polarity of extraction solvents and TPC [25,29]. As far as our literature review could ascertain, the *C. kroumirensis* methanolic extract showed a TPC higher than the one of some other studied *Centaurea* species. The methanolic extract of *C. drabifolia* subsp. *detonsa* exhibited TPC = 40.454 mg GAE/g [33]. Zengin et al. [11] reported that the methanolic extract of *C. pulchella*, *C. drabifolia* subsp. *drabifolia* and *C. lycopifolia* had 55.00, 24.70, and 28.82 mg GAE/g, respectively. Albayrak et al. [1] studied different extracts of *Centaurea* obtained by maceration, soxhlet, and sonication with methanol; the best TPC (12.92 mg GAE/g) was detected in ultrasonic extract of *C. amaena*.

3.2. Antioxidant Properties

The potential of polyphenols to prevent or delay the oxidative degradation of molecules makes them ideal candidates for managing the complications that are associated with oxidative stress. These compounds exhibit antioxidant potency with different ways (i.e., donors of hydrogen, radical scavengers, reducing agents). In this regard, the assessment of antioxidant abilities of different extract of *C. kroumirensis* and *C. sicula* was performed through two universal methods free radical scavenging DPPH and reducing power.

3.2.1. DPPH Scavenging Activity

DPPH assay is widely performed to assess the free radicals scavenging power of herbal extracts or antioxidant compounds [25]. In this test, the antioxidant compounds reduce picrylhydrazyl (purple radical) to picrylhydrazine (pale yellow hydrazine). The discoloration indicates the free radical scavenging ability of tested extract. This ability was evaluated spectrophotometrically by the determination of the IC_{50} values (the concentration required to scavenge 50% of DPPH radicals). IC_{50} value is inversely related to antioxidant capacity of the sample. Table 1, Figure 2 displays the results of antioxidant activity for each extract.

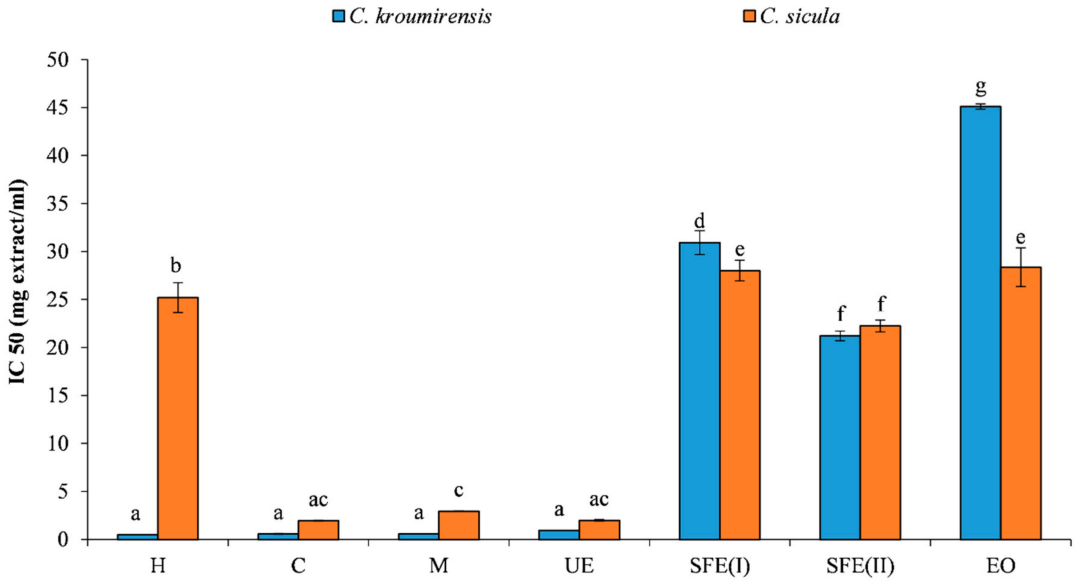

Figure 2. DPPH radical scavenging activity (IC_{50}, mg/mL) determined in extracts obtained from aerial part of two *Centaurea* species (*C. kroumerienis* and *C. sicula*) with different extraction techniques (Different letters indicate significant difference ($p < 0.05$)).

A remarkable difference was noted in the antioxidant activity of the different extracts of *Centaurea* for both species. This might be explained by the different constituents in each extract [29]. According to the literature, several compounds (e.g., cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy, hydroquinone, pyrogallol, gallic acid, p-phenylene diamine, and p-aminophenol) have been well recognized as reducing agents of DPPH due to their hydrogen donation capacity [34]. In the present work, the lowest IC_{50} are recorded in chloroform, ethanol, and methanol extracts for both *entaurea* species that range from 0.58 ± 0.016 to 0.94 ± 0.01 mg/mL and from 1.96 ± 0.05 to 2.94 ± 0.02 mg/mL for *C. kroumirensis* and *C. sicula*, respectively. Interestingly, the hexanic extract of *C. kroumirensis*, which showed the highest antioxidant ability (IC_{50} = 0.49 ± 0.01 mg/mL), does not exhibit the most important TPC. The noteworthy differences could be explained by the synergistic effect of polyphenols extracted in hexanic sample and other non-phenolic antioxidants present in *Centaurea* plant (e.g., vitamin C [35], carotenoids and tocopherols [36], sesquiterpene lactones [37]), which significantly contribute to the free radical scavenging activity [20,38]. The chloroform extract has similar IC_{50} to methanol and ethanol extract of *C. kroumirensis* and *C. sicula*, respectively. The EO and SFE (I-II) extracts for both *Centaurea* species do not exhibit significant antioxidant activities.

3.2.2. Reducing Power Activity

Reducing capacity of plant extract is reflected by the electron-donation ability, which is considered to be an important strategy to behave as an antioxidant. The reducing power was investigated through spectrophotometric detection of Fe^{3+}–Fe^{2+} transformation in the presence of *Centaurea* extract.

The ferrous ion (Fe^{2+}) can be controlled by measuring the optical density of the formed Perl's Prussian blue at 700 nm. Figure 3 illustrates the results of ferric reducing potency. EC_{50} value of each sample was calculated and presented in Table 1. The EC_{50} value is inversely proportional to reducing power.

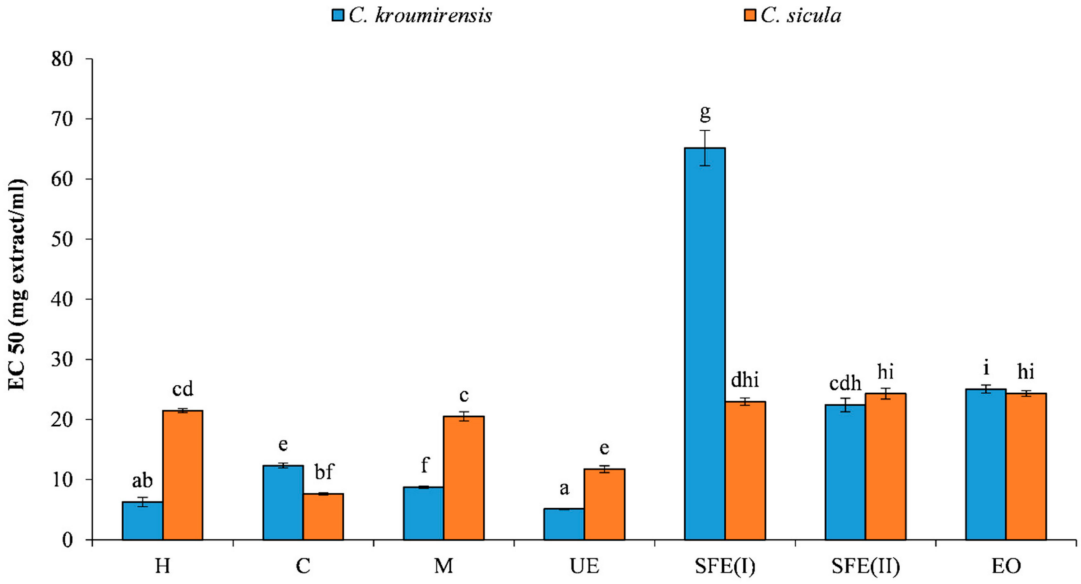

Figure 3. Reducing power (EC_{50}, mg/mL) determined in extracts obtained from aerial part of two *Centaurea* species (*C. kroumerienis* and *C. sicula*) with different extraction techniques (Different letters indicate significant difference ($p < 0.05$)).

In this study, noticeable variation was observed in EC_{50} values within and among the *Centaurea* extracts. Based on the obtained results, the UE and H extracts of *C. kroumirensis* showed the lowest EC_{50} (5.15 ± 0.09 and 6.28 ± 0.77 mg/mL, respectively) and, hence, highest reducing power ability among studied *Centaurea* extracts. Whilst concerning *C. sicula* the lowest EC_{50} (7.62 ± 0.15 mg/mL) was registered for the C extract followed by UE extract with EC_{50} = 11.75 ± 0.58 mg/mL. The reducing power abilities in the studied extracts were ranked in the following order: *C. sicula* hexane < *C. sicula* methanol < *C. sicula* ethanol < *C. sicula* chloroform, for the C. sicula extracts, and *C. kroumirensis* chloroform < *C. kroumirensis* methanol < *C. kroumirensis* hexane < *C. kroumirensis* ethanol, for *C. kroumirensis* extracts.

Similar to the DPPH assay, the EO and SFE (I-II) extracts for both *Centaurea* species did not show a reducing power at least for the concentration used in this study. The SFE (I) of *C. kroumirensis* exhibited the lowest TPC (4.79 ± 0.15 mg GAE/g) and the highest IC_{50} (30.93 ± 1.24 mg GAE/g) and EC_{50} (65.17 ± 2.93 mg GAE/g). These results could be explained by the fact that the supercritical extraction (I) carried out under mild conditions of pressure and temperature (T = 40 °C, P = 10 MP) and for a short time (30 min.), does not allow for obtaining extracts rich in antioxidants.

3.3. Correlation between Total Phenolic Content and Antioxidant Properties

Many reports revealed that, in general, there is a positive correlation between antioxidant abilities of plant extract and its total phenolic content [11,39]. Phenolic compounds, including the flavonoids, phenolic acids, and phenylpropanoids contained in fruits and vegetables, act as reductones that exert antioxidant activity by giving a hydrogen atom or breaking the chain of free radicals [31,40]. Generally, the antioxidant ability of the studied extracts followed the same ranking order with the values of TPC. In the current study, the correlations (Pearson) between antioxidant abilitiy in terms of IC_{50} and EC_{50} for DPPH and reducing power, respectively, and TPC of studied *Centaurea* extracts has been evaluated. Table 2 presents the results.

Table 2. Correlation (Pearson) and relationships between antioxidant capacity and (TPC) in *Centaurea* extracts. Values are with a significance ($p < 0.05$).

Centaurea Specie	Variables	TPC	DPPH (IC_{50})	RP (EC_{50})
C. kroumirensis (Coss.)	TPC	_	−0.758 *	−0.646 *
	DPPH (IC_{50})	_	_	0.676 *
C. sicula L. subsp sicula	TPC	_	−0.959 *	−0.846 *
	DPPH (IC_{50})	_	_	0.802 *

* Correlation is significant at the level.

The average values for IC_{50} exhibited strong (r = −0.959) and good (r = −0.758) negative correlation with TPC in the samples for *C. sicula* and *C. kroumirensis*, respectively. These values indicated that antioxidant ability of *C. sicula* was strongly related to polyphenols that werer extracted in the UE sample. A good negative correlation (TPC–EC_{50}) was recorded for *C. sicula* r = −0.846, however for *C. kroumirensis*, a moderate correlation (r = −0.646) has been noted, which corroborate with the results that were previously discussed for the antioxidant activity of hexanic extract. It could be concluded that polyphenols present most important antioxidant compounds in *C. sicula*, whereas, the aerial part of *C. kroumirensis* is rich in other antioxidants besides polyphenols. Overall, our findings supported the idea that a higher content of phenolic compounds reflected higher antioxidant ability and, inversely, lower TPC lessened the reducing power and DPPH scavenging activities. Additionally, a moderate positive correlation (DPPH (IC_{50})–reducing power (EC_{50})) (r = 0.678 and 0.676) have been recorded for *C. sicula*, and *C. kroumirensis*, respectively. These results corroborated with many reports that revealed a direct correlation between antioxidant abilities and reducing power of plant extract [32,41].

3.4. In Vitro Bioactive Antioxidant Assays

The UE extract of both *Centaurea* plant were selected for the in vitro cell viability test based on the preliminary study of the antioxidant ability. The cytotoxicity of these extracts against human skin fibroblast HS-68 to increasing concentrations has been determined by evaluating viability of tested cell lines after 24 h using MTT assay. The results were expressed in terms of % of vitality cells compared to the control (non-treated cells).

Treatment with UE extracts, in concentrations between 0.02 and 0.32 µg GAE/mL, has not induce any significant decrease in cells viability compared to non-treated cells, as shown in Figure 4. Our results appear to be well supported by previous investigations that report a hardly any or no cytotoxicity and antiproliferative effect of *Centaurea* extracts at low dose against human cell lines, e.g., NALM-6, REH, NB4, KMM-1 [42], HepG2 [43], Caco-2 [44], and A549 [19].

Remarkably, an increase of cell vitality (11.6 ± 1.15%) was observed for cells treated with 0.16 µg/mL of UE extract of *C. sicula*, which indicate a proliferative effect towards this skin fibroblast cell line. Otherwise, the effect of ethanol (EtOH) on HS-68 cells has been evaluated and no effect has been observed. The concentration 0.16 µg/mL have been selected for both *C. sicula* and *C. kroumirensis* in order to evaluate the antioxidant ability in cells. The protective effect test of the UE *Centaurea* extracts after the induction of oxidative stress was carried out for 24 h. Hydrogen peroxide (H_2O_2) is one of the ROS that attack cellular components, leading to cell damage and limiting their viability [45].

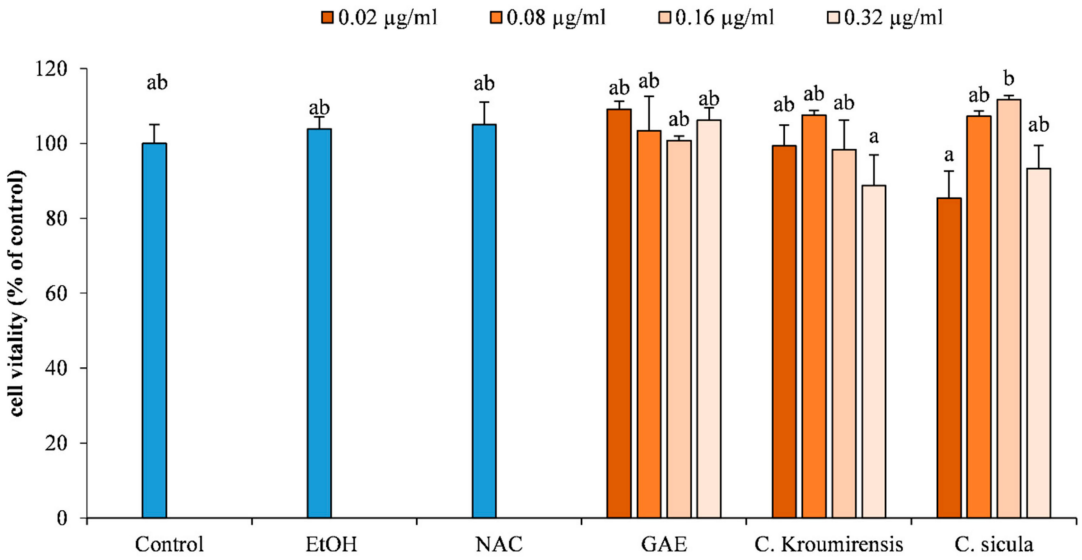

Figure 4. Percentage of vitality of HS-68 fibroblast cells, obtained with MTT test (each data is presented as mean ± SD, n = 5). Cells treated with UE extracts of (*C. kroumerienis* and *C. sicula*) for 24 h to concentrations 0.02, 0.08, 0.16 and 0.32 µg/mL (0.1% < ethanol). Control (cells without treatment) (Different letters indicate significant difference ($p < 0.05$)).

A significant decrease (21.45 ± 5.82%) in cell vitality, determined by MTT test ($p < 0.05$), was noted following the exposure of fibroblast cells to a well-known concentration of H_2O_2 (Control +HP), as shown in Figure 5. However, cells treated with UE of both *Centaurea* extracts exhibited a strong protective effect comparable to that of the natural antioxidant gallic acid (GAE) and to the synthetic antioxidant N-acetilcysteine (NAC). These findings indicate that these extracts possess therapeutic effects, such as inhibition lipid oxidation and the prevention of free radicals' damage, and could be developed as versatile adjuvant in pharmaceutical, food, and cosmetics applications.

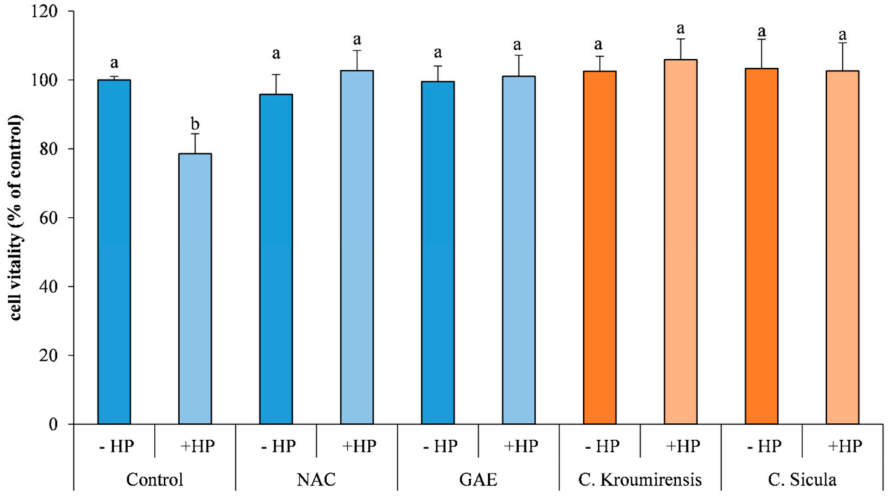

Figure 5. Effect of UE *Centaurea* extracts on HS-68 fibroblast cells exposed to oxidative stress, induced by H_2O_2. HP = hydrogen peroxide, Control cells, NAC = cells pretreated with the synthetic antioxidant NAC, GAE = cells pretreated with gallic acid, cells pretreated with UE extract of *C. kroumerienis*, cells pretreated with UE extract of *C. sicula.* (Different letters indicate significant difference ($p < 0.05$)).

4. Conclusions

The present study revealed noticeable significant variations in the antioxidant ability and TPC among the studied extract of *Centaurea kroumirensis (Coss.)* and *Centaurea sicula* L. subsp *sicula*. The UE extracts are rich in TPC for both *Centaurea* species, and they exhibited considerable antioxidant activity in the tested methods. Both studied extract showed a strong protective effect in human fibroblast cell that was exposed to oxidative stress. Taken together, these results indicate that this extract could possess therapeutic effects such as inhibition lipid oxidation and prevention of radical damage. For the above-mentioned reasons, *Centaurea* species could be considered as a potential natural candidate for designing novel phytopharmaceutical or for nutritional application as an ingredient in the formulation of new nutraceuticals. Further studies are needed in order to isolate and identify the antioxidants in these extracts responsible activities observed.

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