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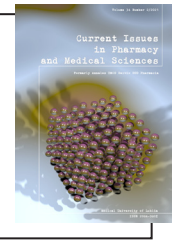

Antioxidant and antiproliferative activities of the *n*-butanol extract of *Centaurea maroccana* Ball aerial parts

Imen Aissous[1], Mouad Benrebai[1], Ercan Cacan [2], Berkel Caglar[2], Ramazan Erenler[3], Souad Ameddah[1], Samir Benayache[4], Fadila Benayache[4], Chawki Bensouici[5]*

[1] Laboratory of Biology and Environment, Faculty of Sciences of Nature and Life, University of Mentouri Constantine 1, Constantine, Algeria
[2] Tokat Gaziosmanpasa University, Department of Molecular Biology and Genetics, Tokat, Turkey
[3] Tokat Gaziosmanpasa University, Faculty of Arts and Sciences, Department of Chemistry, Tokat, Turkey
[4] Valorization of Natural Resources Unit, Bioactive Molecules and Physicochemical and Biological Analysis (VARENBIOMOL), University of Mentouri Constantine 1, Constantine, Algeria
[5] Biotechnology Research Center (CRBt), Ali Mendjli, Constantine, Algeria

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ABSTRACT

The aim of the present study is to evaluate the total phenolic contents, antioxidant and anti-proliferative activities of the *n*-BuOH extract of *Centaurea maroccana* (BECM). The total phenolic and flavonoid of the butanolic extract of the plant were assessed by using Folin-Ciocalteau and aluminium chloride colorimetric assays, respectively, and the quantitative estimation of total flavonoids and phenols revealed the richness of the extract in these compounds. Antioxidant activity was evaluated using standard lab colorimetric methods, while the anti-proliferative activity was evaluated using sulforhodamine B (SRB) assay against C6 (*Rattus norvegicus* brain glioma) and HT29 (*Homo sapiens* colorectal adenocarcinoma) cell lines. The *n*-butanolic extract of *Centaurea maroccana* showed a strong antioxidant activity through DPPH, $ABTS^{\bullet+}$, DMSO alkalin, Reducing power, β-Carotene-linoleic acid and CUPRAC assays. Furthermore, the anti-proliferative activity against C6 and HT29 of *Centaurea maroccana* exhibited a high effect by the decrease in viability of both cancer cell lines. Our results suggest a possible use of *Centaurea maroccana* as a source of natural antioxidant and chemo-preventive agents against cancer.

INTRODUCTION

Antioxidants are inhibitors of the oxidation of an oxidizable substrate while at relatively low concentrations when compared with the substrate [1]. Preventing diseases caused by free radicals and preserving human health are the most vital roles of antioxidants [2]. Antioxidants, especially phenolic compounds, are abundant in several medicinal plants [3], where polyphenols are the most common present secondary metabolites [4]. In recent times, phenolic compounds of natural origin have been investigated, as their indirect antioxidant activity may lead to oxidative stress reduction [5].

About the Mediterranean zone and West Asia, the genus *Centaurea* is represented by approximately 500 species [6]. In Algeria alone, 47 species of this genus are common [7]. In some countries, various species of *Centaurea* plant have been exploited in folk medicine to treat several diseases [8]. This is the outcome of recognizing their significant biological activities [9]. Among these are: antioxidant activity [10], anti-inflammatory [11], and antimicrobial [12] antiviral, antibacterial, and antifungal [13] and cytotoxic [14] activities. In addition, several *Centaurea* species are considered to be valuable sources of bioactive secondary metabolites, particularly, flavonoids and sesquiterpene lactones [15] – which are significant chemotaxonomic indicators [16].

Centaurea maroccana is an endemic herb in the north of Algerian Sahara [17], thus, the aim of the present study was to assess the *in vitro* antioxidant and the anti-proliferative activities of an *n*-BuOH extract of *Centaurea maroccana* (BECM) and to correlate the results with the total phenolic content and the amount of flavonoids through colorimetric methods.

* **Corresponding author**
e-mail: chawkiislam@yahoo.fr

MATERIALS AND METHODS

Plant material

Centaurea maroccana was collected from the area of Biskra in the South of Algeria and authenticated by Prof. M. Kaabeche (Biology department, University of Setif, Algeria) according to Quezel and Santa [18]. A voucher specimen (CCM12/04/02) was then deposited in the Herbarium of the Biology Department of Mentouri University of Constantine.

Extraction

Air-dried aerial parts (leaves and flowers, 2700 g) of *Centaurea maroccana* were powdered (slight grinding with controlled temperature, up to 35°C) and macerated at room temperature with MeOH-H_2O (80:20, v/v) three times (24 hours for each time). The filtrates were combined, concentrated under reduced pressure (up to 35°C), diluted in H_2O (1000 ml) under magnetic stirring and maintained at 4°C for one night to precipitate a maximum of chlorophylls. After filtration, the resulting solution was successively extracted with solvents with increasing polarities: chloroform ($CHCl_3$), ethyl acetate (EtOAc) and n-butanol (*n*-BuOH). The organic layers were dried with anhydrous Na_2SO_4, filtered and concentrated under vacuum at room temperature to obtain $CHCl_3$ (12.5 g, yield: 0.46%), EtOAc (20 g, yield: 0.74%) and *n*-BuOH (40 g, yield: 1.48%) extracts. A part of the *Centaurea maroccana n*-BuOH extract (designated BECM) was used in the current study.

Spectral measurements and chemicals used

The measurements and calculations of the activity results were established by using a 96-well microplate reader (PerkinElmer Multimode Plate Reader EnSpire, USA) at the National Center of biotechnology Research. The chemical products and reagents used were: Folin-ciocalteu's reagent (FCR), 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated-hydroxylanisole (BHA), Butylatedhydroxyltoluene (BHT), α-Tocopherol, β-carotene, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40), Neocuproine,2,2›-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid)diammoniumsalt (ABTS), Trichloroacetic acid (TCA), Ethylenediaminetetraacetic acid (EDTA), Nitro blue tetrazolium (NTB), Diméthyl sulfoxyde (DMSO), Sulforhodamine B (SRB), and were obtained from Sigma Chemical Co. j(Sigma-Aldrich GmbH, Stern-heim, Germany), while Sodium Carbonate, Aluminum Nitrate, Iron (III) chloride ($FeCl_3$), Iron (II) chloride, Sodium bicarbonate, Potassium acetate, were obtained from Biochem Chemopharma. All other chemicals and solvents were of analytical grade.

Determination of total bioactive compounds

Determination of total phenolic content (TPC)

The total phenolic content in the extract was determined spectrophotometry via Folin-Ciocalteu reagent according to the method of Muller *et al.* [19], with a minor modification. In doing so, 1 mg of extract was dissolved in a volume of 1 mL of methanol, then 20 µL of extract at various concentrations were mixed with 100 µL of Folin-Ciocalteu reagent diluted to 1:10 and 75 µL of sodium carbonate solution (75 g/L) in the wells of a 96-well microplate in the dark at room temperature. After 2 h, the absorbance was measured at 740 nm. A blank is prepared in the same way by replacing the extract with the solvent used (Methanol). Total polyphenols was quantified by employing a gallic acid calibration curve at different concentrations. The total content of phenolic compounds was expressed as micrograms of gallic acid equivalents per milligram of extract (µg EAG/mg). All bio-activity measurements were carried out using a 96-well microplate reader (Perkin Elmer Enspire, USA).

Determination of total flavonoid Content (TFC)

The establishment of total flavonoid content of the extract was based on the method described by Topçu *et al*. [20], with slight modifications for determination on a 96-well microplate. Accordingly, 1mg of extract was dissolved in a volume of 1 mL of methanol. Subsequently, 130 µL (MeOH), 10 µL of potassium acetate (CH_3COOK) prepare in water and 10 µL of the aqueous solution of aluminium nitrate (Al $(NO_3)_2$, $9H_2O$) was added to a volume of 50 µL of extract at various dilutions. The absorbance was read at 415 nm, after incubation at room temperature for 40 min. A blank sample was prepared by replacing the reagents with methanol (50 µL extract + 150 µL methanol). The quantification of flavonoids was deduced from a calibration range established with quercetin, as a positive control. The results were evaluated as micrograms of quercetin equivalents per milligram of extract (µg EQ/mg).

Determination of antioxidant activity

DPPH$^{\cdot}$ scavenging assay

The free anti-radical activity was determined spectrophotometry via DPPH assay [21]. Briefly, 40 µL of MeOH extract solution at various concentrations was added to 160 µL of DPPH (0.1 mM). The reaction mixture was then shaken vigorously and the absorbance of the remaining DPPH was measured at 517 nm after 30 min. BHA and BHT were used as antioxidant standards for activity comparison. The scavenging capability of DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

- Abs control is the absorbance of the reaction containing only the reagents.
- Abs Extract is the absorbance of the reaction containing the reagents and the extract.

The results were given in the form of IC_{50} value (µg/mL) corresponding to the concentration of 50% inhibition.

ABTS cation radical Assay

The $ABTS^{\cdot+}$ scavenging activity was derived according to the method of Re *et al*. [22]. The cation radical ABTS was generated by mixing 7 mM of an aqueous solution of ABTS with 2.45 mM of potassium persulfate, the whole was then stored protected from light and at room temperature for 16h before use. The solution obtained was subsequently diluted with ethanol to obtain an absorbance of 0.700±0.025

at 734 nm. After this, 160 µL of ABTS solution was added to 40 µL of methanol extract solution at different concentrations. After 10 min, the absorbance was measured at 734 nm. Methanol was used as a control; BHA and BHT were used as antioxidant standards for the comparison of extract activity.

The scavenging capacity of the $ABTS^{\bullet+}$ was calculated using the following equation:

$$ABTS^{\bullet+} \text{ Scavenging effect } (\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Superoxide radical scavenging assay by alkaline DMSO

The scavenging activity of superoxide radicals by alkaline DMSO was obtained by the method of Rao and Kunchandy [23], with the reduction of NBT by superoxide being assessed in the presence and in the absence of extracts. In accomplishing this, the reaction mixture containing NBT (1 mg/mL in distilled water) and 40 µL of extract at various dilutions were added to 130 µL of alkaline DMSO (1 mL of DMSO containing, 5 mM NaOH in 0.1 mL of water) to give a final volume of 200 µL and the absorbance was measured at 560 nm using a microplate reader (Perkin Elmer Enspire, USA). The decrease in absorbance at 560 nm with antioxidants indicates the consumption of the superoxide generated, and the percentage of scanning was calculated using the following formula:

$$\text{Inhibition } (\%) = \frac{A_{Sample} - A_{Contro}}{A_{Sample}} \times 100$$

Reducing power assay

The reducing power of the tested extract was established according to the method of Aicha Bouratoua [24]. In order to determine the reducing power activity, 10 µL of serial diluted sample were added into a 96 well round-bottomed plate. Following this, 40 µL of 0.2 M phosphate buffer (pH 6.6) and 50 µL of potassium ferricyanide (1%), were added to each well and the plate was incubated at 50°C for 20 min. Finally, 50 µL of TCA (10%) and distilled water (40 µL) and 10 µL of ferric chloride (0.1%) were added into each well in order to measure the reducing power activity. After this, the absorbance was measured in a microplate reader (Perkin Elmer Enspire, USA) at 700 nm. Note: higher absorbance of the reaction mixture indicates greater reducing power.

***β*-carotene/linoleic Acid bleaching assay**

The bleaching activity of *β*-carotene was evaluated using the method described by Marco [25]. A mass of 0.5 mg of *β*-carotene dissolved in 1 ml of chloroform was added to a volume of 25 µL of linoleic acid and 200 mg of Tween 40, giving an emulsifying mixture. After evaporation of the chloroform in vacuo, 50 mL of distilled water saturated with oxygen was added with vigorous stirring. The absorbance of the solution was then adjusted between 0.8-09 nm. 160 µL of this solution was added to 40 µL of extract solution at different concentrations. Absorbance was measured at 470 nm using a 96-well microplate reader (Perkin Elmer Enspire, USA). The emulsion system was subsequently incubated for 2 h at 50ºC, while a blank devoid of *β*-carotene, was prepared for the background subtraction. BHA and BHT were used as standards. The bleaching rate (R) of *β*-carotene was calculated according to the following equation:

$$R = (\ln a/b)/t$$

The antioxidant activity (AA) was derived in terms of percent of inhibition relative to the control, using the following equation:

$$AA\ (\%) = \frac{R_{Control} - R_{Sample}}{R_{Control}} \times 100$$

Cupric reducing antioxidant capacity (CUPRAC)

The antioxidant activity by cupric reduction was assessed according to the method of Apak *et al*. [26]. A reaction mixture containing 40 µL of the extract at different concentrations, 50 µL of a copper (II) chloride solution, 50 µL of neocuproin alcoholic solution and 60 µL of ammonium acetate aqueous buffer at pH 7 was placed in each well of a 96-unit microplate. After 30 min, the absorbance was recorded at 450 nm. The results were calculated as $A_{0.5}$ (µg/mL) corresponding to the concentration indicating 0.50 of absorbance. BHA and BHT were used as antioxidant standards.

Determination of *in-vitro* Antiproliferative Activity
Cell Culture

C6 (*Rattus norvegicus* brain glioma) and HT29 (*Homo sapiens* colorectal adenocarcinoma) cell lines were cultured in DMEM (Sigma-Aldrich; St. Louis, MO). All media were supplemented with 10% fetal bovine serum (FBS) and 1% Pen/Strep. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2. The plant extract was dissolved in double distillated water, and stock solutions were filtered using 0.45 µm syringe filters (Minisart, Sartorius Stedim Biotech, Goettingen, Germany). Stock solutions were kept at 4°C for further use and diluted to desired concentrations with media before treatment.

Cytotoxicity Assay

Sulforhodamine B (SRB) Assay was used to determine percent cell viability of cell lines (C6, HT29) treated with various concentrations of *Centaurea maroccana* extract as described in Vichai and Kirtikara [27]. Briefly, 7500 cells were seeded per well in a 96-well plate (flat bottom). After 24 h incubation, cells were treated with various concentrations of the extract (*n*-butanolic extract of *Centaurea maroccana*) and further incubated for 72h. The cells were then fixed with 10% trichloroacetic acid (TCA) for 1.5 hours at 4°C and washed with ddH_2O for 4 times. Following this, 0.4% SRB dye (dissolved in 1% acetic acid) was added to each well in the dark and the plates were incubated for an additional 30 min at room temperature. After washing with ddH_2O for 4 times, the plates were dried and the dye was solubilized by incubating the cells with 10 mM Tris base solution for 15 minutes on a shaker. Spectrophotometric reading was then performed at 565 nm using a Multiskan FC microplate reader (Thermo Scientific, MA, USA). OD values were used for analysis.

Statistical analysis

All data on all activity tests were the average of triplicate analyses. The data were recorded as mean ± standard deviation (SD). Significant differences between means were determined by Student's-test, p values <0.05 were regarded as significant.

RESULTS AND DISCUSSION

Total phenolic content

Phenolic compounds are secondary metabolites that have antioxidant, anti-inflammatory, antimicrobial, and anti-cancer activities [28]. Research indicates that phenolic content correlates with antioxidant activity [29]. The antioxidant activity of phenolic contents comes about due to their redox properties that permit them to scavenge radicals, metal chelators, singlet oxygen quenchers, hydrogen donors, and reducing agents [30]. Hence, it is important to quantify and assess the effect of the phenolic content on the antioxidant activity in our extract.

In our work, the total phenolic content of BECM was determined using the Folin-Ciocalteu reagent and expressed as micrograms of gallic acid equivalents per milligram of extract. The derived valued was calculated from the linear regression equation of a standard curve ($y = 0.0056x+0.0948$, $R^2 = 0.9953$). Results show that the *n*-butanol extract of *Centaurea maroccana* (BECM), in particular, had high phenolic content at 242.30±1.44 µg GAE/mg.

Total flavonoids content

Flavonoids are naturally occurring polyphenols existing in almost all plant resources [31]. The high antioxidant potential of flavonoids lies in their ability to eliminate free radicals and injurious ROS that arise from several cellular activities and lead to oxidative stress [32]. In the present study, the concentrations of flavonoids was determined using the aluminum chloride colorimetric method and was calculated from the linear regression equation of a standard curve of quercetin ($y = 0.0098x + 0.0623$, $R^2 = 0.985$). The quantification is expressed as micrograms quercetin equivalents per milligram of extract. The results reveal that the amount of flavonoids in the n-butanol extract of BECM is 23.34±1.28 µg QE/mg.

Antioxidant properties

In this study, the antioxidant activity of an *n*- butanol extract of *Centaurea maroccana* was established through by applying six *in-vitro* assays: DPPH radical scavenging, ABTS cation radical scavenging, superoxide radical scavenging assay by alkaline (DMSO), *β*-carotene-linoleic acid bleaching, cupric reducing antioxidant capacity (CUPRAC) and reducing power activity.

The DPPH assay has frequently been used for assessing the anti-oxidative potential of natural products as it is considered a rapid and least cost method [33]. DPPH at room temperature is a stable free-radical that receives an electron/hydrogen radical to become a stable diamagnetic molecule [34]. Antioxidants interact with DPPH, changing its deep violet color to yellow.

The ability of the BECM extract to scavenge the DPPH radical is shown in Table 1. The reference compounds used in this test are BHT and BHA. It can be seen that a significant decrease ($p < 0.05$) in the concentration of DPPH radicals was exhibited by the extract and standards. In our work, the IC_{50} value of BECM extract was found to be 12.09±0.54 µg/mL. This figure is higher than that of the BHT standard (22.32±1.19 µg/mL), but lower than the IC_{50} of BHA at 5.73±0.41 µg/mL. Thus, these results reveal that the n-butanol extract has a high DPPH radical scavenging effect that is comparable with the standards.

Table 1. DPPH radical scavenging activity of BECM and the standards butylated hydroxy toluene (BHT) and butylated hydroxyl lanisole (BHA)

Concentration µg/mL	% Inhibition in DPPH Assay		
	BECM	BHT	BHA
3.125	17.12±1.57	11.69±1.88	28.95±1.16
6.25	26.77±3.42	22.21±1.30	54.33±1.59
12.5	50.35±3.03	37.12±1.80	76.76±1.65
25	84.70±1.49	52.63±2.70	84.09±0.35
50	84.95±0.23	56.02±0.53	87.53±0.82
100	85.76±0.95	83.60±0.23	87.73±0.15
200	86.31±1.67	87.28±0.26	88.43±0.23
IC_{50} µg/mL	12.09±0.54	22.32±1.19	5.73±0.41

The ABTS radical scavenging method is a most dependable method for determining free radicals destruction [35]. The $ABTS^{\bullet+}$ chromophore is generated during the reaction of ABTS and potassium persulfate through the transformation of ABTS into a radical cation. The color of this radical cation is blue and absorbs light at 734 nm [36].

Table 2 shows that $ABTS^{\bullet+}$ cation radical was significantly inhibited at all concentrations tested. However, at the high concentration of 200 µg/mL, the percentage inhibition by the BECM reached 93.05±0.65%. This outcome is similar to BHT and BHA (96.68±0.39% and 95.39±2.62%, respectively). Hence, good $ABTS^{\bullet+}$ free-radical scavenging activity was revealed by BECM at IC_{50} = 15.13±0.87 µg/mL, in relation to the standards BHT (IC_{50} = 1.29±0.30 µg/mL) and BHA (IC_{50} = 1.81±0.10 µg/mL).

Table 2. Free radical scavenging activity of *n*-butanol extract of *Centaurea maroccana* on ABTS (2, 2-azinobis-3-ethylbenzothiazoline-6-sulfonic)

Concentration µg/mL	% Inhibition in ABTS Assay		
	BECM	BHA	BHT
3.125	12.52±2.61	83.42±4.09	59.22±0.59
6.25	24.68±1.70	93.52±0.09	78.55±3.43
12.5	47.49±3.09	93.58±0.09	90.36±0.00
25	73.77±2.88	93.63±0.16	92.18±1.27
50	90.36±1.76	93.63±0.95	93.37±0.86
100	92.73±1.42	94.20±0.90	94.87±0.87
200	93.05± 0.65	95.39±2.62	96.68±0.39
IC_{50} µg/mL	15.13±0.87	1.81±0.10	1.29±0.30

BHT and BHA were used as standards

The superoxide radical is the precursor of more reactive species and is recognized as one of the most damaging species for cellular constituents [37]. The super oxide scavenger capacity of antioxidants present in the extract is determined by the decrease in absorbance at 560 nm. Table 3 shows that the BECM inhibits the formation of the super oxide anion radical generated in vitro by the DMSO alkaline system in a dose-dependent manner. The maximum inhibition of NBt reduction by super oxide (91.57±0.51%) was observed at the high concentration of 200 µg/mL and was comparable to Tannic acid and α-Tocopherol standards (97.54±0.68%, 96.86±1.53%, respectively). Of significance, however, is that the butanolic extract of *Centaurea maroccana* has a stronger super oxide scavenging activity (IC_{50} = 4.50±0.33) than the two standards used (IC_{50} = < 3.125 and < 3.125).

Table 3. Superoxide radical scavenging assay by alkaline DMSO of butanolic extract of *Centaurea maroccana*

Concentration µg/mL	% Inhibition in DMSO alcalin assay		
	BECM	Tannic acid	α-Tocopherol
3.125	47.73±4.47	83.58±1	70.09±1.84
6.25	64.83±3.77	88.35±0.61	79.07±2.54
12.5	74.40±2.73	92.66±0.42	85.1±1.36
25	80.66±1.08	94.98±0.85	89.48±0.88
50	85.97±1.03	96.53±0.23	94.76±0.28
100	88.28±0.52	96.95±0.81	96.62±0.29
200	91.57±0.51	97.54± 0.68	96.86±1.53
IC_{50} µg/mL	4.50±0.33	< 3.125	< 3.125

Tannic acid and α-Tocopherol were used as standards

Reducing power activity is used to assess the potential and the capacity of natural antioxidants to donate electrons [38], and there is a correlation between both the reducing properties and the existence of reductones [39]. These reductones convert yellowed coloured ferric iron (Fe^{3+}) to blue-green coloured ferrous iron (Fe^{2+}). Hence, reducing activity is determined by measuring the formation of Pearl's Prussian blue at 700 nm. In this study, BECM had the power to reduce ferric ferricianide through increasing the absorbance via concentration. In our experiment, it exhibited an absorbance (1.11±0.09) at the concentration of 50 µg/mL, while the positive references BHT, BHA and Ascorbic Acid exhibited an absorbance of 0.43±0.02, 3.53±0.19, 3.87±0.27, respectively, at the same concentration (Table 4). Thus, this extract ($A_{0.50}$ = 14,19±0,96 µg/mL) has a strong effect compared with BHA ($A_{0.50}$ = >50), BHA ($A_{0.50}$ = 8.41±0.67) and Ascorbic Acid ($A_{0.50}$ = 9.01±1.46).

We used the β-carotene-linoleic acid method to detect the inhibition level of lipid peroxidation [40]. In the β-carotene/linoleic acid system, the oxidation of linoleic acid produces peroxyl free radicals owing to the abstraction of a hydrogen atom from the diallylic methylene groups of linoleic acid [41]. The resulting peroxy-free radicals participate in an emulsion attack upon the chromophore β-carotene, resulting in bleaching and loss of yellow color. The presence of antioxidant in this reaction mixture brings about the scavenging of radicals and the suppression of oxidation [28]. The results as shown in Table 5, and demonstrate that the lipid peroxidation inhibitory ability of the BECM increases significantly (p 0.05) with increasing concentration. Here, the bleaching inhibitory activity of the extract tested at the highest concentration of 200 µg reached a percentage of 83.48±3.97%, whereas the positive standards of BHT reached 95.28±3.25%, while that of BHA reached 99.76±0,91. Thus, BECM has revealed itself to have interesting antioxidant activity in β-carotene bleaching, having an IC_{50} = 11.08±0.30 µg/mL, in comparison to the standards of BHT and BHA, which are in the following order: 1.05±0.01 µg/mL, 0.90±0.02 µg/mL, respectively.

Table 4. Antioxidant activity of *Centaurea maroccana* by reducing power assay

Concentration µg/mL	Absorbance			
	BECM	BHA	BHT	Ascorbic Acid
0.78125	0.13±0.00	0.09±0.00	0.07±0.00	0.09±0.00
1.5625	0.16±0.00	0.11±0.01	0.08±0.00	0.11±0.00
3.125	0.17±0.02	0.18±0.02	0.10±0.01	0.16±0.01
6.25	0.31±0.02	0.36±0.04	0.13±0.02	0.33±0.04
12.5	0.44±0.02	0.78±0.07	0.22±0.04	0.76±0.16
25	0.71±0.08	1.74±0.07	0.28±0.05	2.02±0.23
50	1.11±0.09	3.53±0.19	0.43±0.02	3.87±0.27
$A_{0.50}$ µg/mL	14.19±0.96	8.41±0.67	>50	9.01±1.46

BHT, BHA and Ascorbic Acid were used as standards

Table 5. Antioxidant activity of *Centaurea maroccana* by the *β*-carotene-linoleic acid.

Concentration µg/mL	% Inhibition in β-carotene assay		
	BECM	BHA	BHT
3.125	27.99±6.82	84.23±1.14	81.14±0.84
6.25	38.57±4.12	90.11±0.68	86.0.9±1.04
12.5	51.40±5.36	94.59±0.77	87.52±4.24
25	69.15±2.36	96.09±0.02	91.67±0.52
50	77.19±3.07	97.35±1.08	94.11±0.42
100	83.34±2.28	99.59±0.14	94.41±0.32
200	83.48±3.97	99.76± 0.91	95.28±3.25
IC50 µg/mL	11.08±0.30	0.90±0.02	1.05±0.01

BHT and BHA were used as standards

The CUPRAC assay necessitates the use of comparatively standard equipment, where it is applied extensively to determine the antioxidant abilities of plant extracts, and gives quick and repeatable results [42]. The method is founded on the follow-up of the reduction in the augmented absorbance of Neocuproine (Nc) copper (Cu^{2+}) [Nc_2_Cu^{2+}], wherein the existence of an antioxidant agent reduces the complex (copper-neocuproine). This reaction is measured at 450 nm. In our work, *Centaurea maroccana* extract revealed a strong CUPRAC reducing antioxidant ability with an $A_{0.50}$ value equal to 9.80±0.43 µg/mL. This is similar to the standards used, with BHT at $A_{0.50}$ = 9.62±0.87µg/mL, and BHA at $A_{0.50}$ = 3.64±0.19µg/mL. At 200 µg/mL concentration, roughly same reducing activity was observed via extract and BHA at 3.48±0.13% and 3.93±0.01%, respectively, while BHT was 2.32±0.28% (Table 6).

Table 6. Antioxidant activity by the cupric reducing antioxidant capacity (CUPRAC) assay of *Centaurea maroccana* butanolic extract

Concentration µg/mL	Absorbance		
	BECM	BHA	BHT
3.125	0.23±0.00	0.46±0.00	0.19±0.01
6.25	0.39 ±0.06	0.78±0.01	0.33±0.04
12.5	0.59±0.02	1.34±0.08	0.66±0.07
25	1.02 ±0.06	2.36±0.17	1.03±0.07
50	1.76±0.14	3.45±0.02	1.48±0.09
100	2.49±0.29	3.76±0.03	2.04±0.14
200	3.48±0.13	3.93±0.01	2.32±0.28
$A_{0.50}$ µg/mL	9.80±0.43	3.64±0.19	9.62±0.87

In summary, it can be deduced that the high levels of antioxidant activity exhibited by BECM were due to its richness of phenolic components and flavonoids, wherein phenolic components display antioxidant activity and play a significant role in stabilizing lipid peroxidation [43].

Antiproliferative Activity

After performing cytotoxicity screening using SRB Assay, we observed that n-butanolic extract of *C. maroccana* (BuOH ext.) significantly decreased percent viability of both cell lines (C6-glioma cell line, HT29-colorectal adenocarcinoma cell line) with increasing concentrations in the range of 50-300 µg/mL (Fig. 1), and with highly similar cytotoxic effect. At 300 µg/mL concentration, cell viability was less than 50% compared to control cells (no treatment) in both C6 and HT29.

We then examined the effect of a combined treatment of BECM and cisplatin, a common chemotherapeutic drug, on these cancer cell lines. The outcome of this was that the combination of cisplatin with BECM treatment caused a slight decrease in cell viability for both cell lines compared to cisplatin treatment alone.

Boxplots showing percent cell viability of cell lines treated with indicated *n*-butanol extract concentrations (BuOH ext.) and/or cisplatin. Cytotoxicity was measured using SRB assay and 5 µM cisplatin was used in these experiments. Blue boxplots indicate combination treatments with cisplatin.

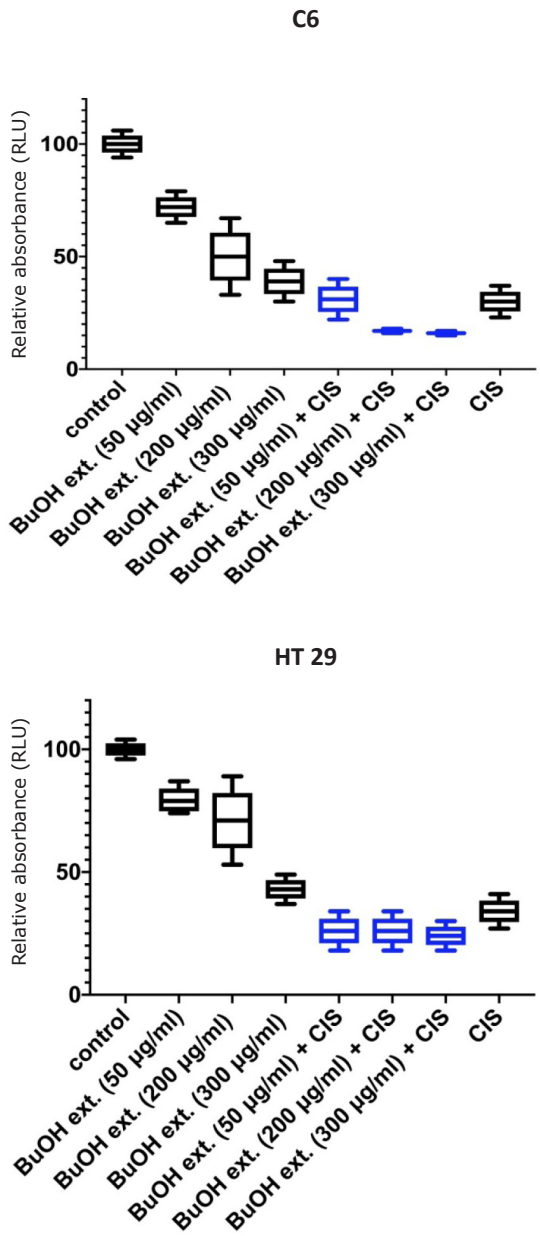

Figure 1. Cytotoxicity of *n*-butanol extract of *Centaurea maroccana* on C6 and HT29 cell lines

CONCLUSION

In conclusion, the results of this study indicate that the *n*-butanolic extract of *Centaurea maroccana* possesses high total phenolic content and exhibits strong antioxidant activity through scavenging several free radicals. This synergistic antioxidant ability owing to the phenolic compounds present in the plant emphasizes the good correlation among antioxidant properties and phenolic content. Furthermore, we observed that the n-butanolic extract of *Centaurea maroccana* significantly decreases the percent viability of both cell lines (C6–glioma cell line, HT29–colorectal adenocarcinoma cell line) with increasing concentration. The present investigation showed that *Centaurea maroccana* can be considered as a source of bioactive compounds that could be used in the pharmaceutical and food industries. Further studies focusing on the isolation and identification of the principle bioactive molecules of antioxidant and antiproliferative activity are, however, needed.

ORCID iDs

Chawki Bensouici https://orcid.org/0000-0003-4612-4642

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