Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 3, pp. 253–263
DOI: 10.31883/pjfns/122040
http://journal.pan.olsztyn.pl

Original research article
Food Quality and Functionality Section

Evaluation of the Antioxidant and Cytotoxic Activities on Cancer Cell Line of Extracts of Parasitic Plants Harvested in Tunisia

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Key words: weeds, resource, wild edibles, biodiversity food, DPPH, phenolics

Weeds thrive in agricultural environments. However, in certain areas of the world, they are consumed by humans as food, and they can represent a source of valuable active ingredients of ethnomedical interest. In this study, three North African parasitic plants of the Orobanchaceae family, *Cistanche violacea*, *Orobanche crenata* and *Orobanche lavandulacea*, normally considered as weeds, were studied to compare their biological activities. The antioxidant activity of hexane, ethyl acetate, acetone, methanol, and water extracts obtained by sequential maceration was estimated by DPPH, ABTS and FRAP assays. The metabolic profiles of aqueous extracts were studied by ^{1}H NMR spectroscopy. The oils extracted using hexane as solvent were analyzed by GC-FID and HPLC-DAD to characterize the fatty acid profile. The cytotoxic effect of the oil from the parasitic plants was also studied on B16F10 melanoma cancer cells. All extracts showed antioxidant activity and differences between extracts obtained using different solvents as well as for different plants have been highlighted. The highest total phenolic content was determined in *O. crenata* acetone extract (3.53±0.20 mmol GAE/g) and in *O. lavandulacea* methanol extract (2.29±0.02 mmol GAE/g). ^{1}H NMR spectroscopy revealed the presence of amino acids, organic acids and carbohydrates. The major fatty acids in the oils were 18:2 *n*-6, 16:0, 18:1 *n*-9, and 18:3 *n*-3. *O. lavandulacea* revealed to be a highly cytotoxic species for B16F10 melanoma cells. These data suggest that, despite the extreme climate conditions and the damage that can result, the studied desert parasitic plants can represent a novel and precious source of metabolites, with promising biological activities.

INTRODUCTION

The term 'weed' defines a plant that does not have any function considered useful for agricultural production and causes damage to existing plants. The harmfulness of weeds appears mainly in the following aspects: competition for solar radiation, water and nutrients, depreciation of the crop in the presence of fragments of pests, development of pests and diseases. Sometimes the term 'plant parasite' is incorrectly used as a weed equivalent. Thus, parasitic plants are plants that are often deemed undesirable. In the most disadvantaged areas of the world there is a need to look for less expensive food sources [Khattak *et al*., 2006]. Conventional vegetables grown in fields are expensive for most people. Parasitic plants could prove to be a good alternative for expensive vegetables [Ceccanti *et al*., 2018]. There is evidence dating back many centuries about the use of some parasitic plants as food in times of famine or as a source of precious drugs widely used in folk medicine. Nevertheless, very little is known about the richness of secondary metabolites of parasitic plants and their potential use in human nutrition.

In North Africa, especially in Tunisia, holoparasitic plants are restricted to a few botanical families: Cuscutaceae [El Mokni *et al*., 2016], Cynomoriaceae [Ben Attia *et al*., 2018; Zucca *et al*., 2019], Orchidaceae [El Mokni *et al*., 2010], Rafflesiaceae and Orobanchaceae [El Mokni *et al*., 2015]. These plants lack of chlorophyll and depend on their hosts for carbon and/or mineral nutrition. However, parasitic plants can cause a serious threat to several food crops and spontaneous vegetation, requiring careful monitoring of their agronomical, ecological and economic impacts [Nickrent & Musselman, 2004]. Orobanchaceae is an unconventional edible parasitic plant family, similar to the asparagus [Nada & El-Chaghaby, 2015], which is part of the flora of Tunisia. Despite they can negatively impact food crops, these plants also have some medicinal uses [Zhang *et al*., 2018]. Contemporary pharmacological studies, in fact, have progressively validated the traditional uses of Orobanchaceae [Wang *et al*., 2017].

In southern Tunisia, three common holoparasitic plant species, belonging to the Orobanchaceae, are: *Cistanche violacea* (Desf.) Hoffmanns & Link, *Orobanche crenata* Forssk, and *Orobanche lavandulacea* Rchb. *Cistanche violacea*, synonym of *Phelypaea violacea* Desf. is a species of angiosperms Eudicotyledonous, endemic to North Africa (*i.e.* Tunisia, Libya, Algeria, Morocco, Western Sahara, Egypt, Saudi Ara-

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bia) [Baba Aissa, 2011]. Ecologically, this species is localized on heavy and salty soils. It is an obligate parasite species of Chenopodiaceae and Zygophyllaceae. In Saharan areas, *C. violacea* parasites *Zygophyllum cornutum* and *Limoniastrum guyonianum*. In Libya, the fat and fleshy underground part is consumed during famine, and the Tuaregs eat this species as asparagus. The genus *Cistanche* is considered the most valuable species of the Orobanchaceae with a strong resistance to harsh environmental conditions.

Orobanche crenata is a parasitic herbaceous species, without chlorophyll and hermaphrodite. At the end of the flowering (Spring season) the plant becomes russet and the flowers become more consistent. It is more common in countries adjacent to the Mediterranean. It extends east to Pakistan and India, and north to Europe. The host range of *O. crenata* is restricted to Fabaceae and Apiaceae and to a relatively sporadic occurrence in a few other families, such as Asteraceae and Cucurbitaceae. The wild hosts are mainly in these same families.

Orobanche lavandulacea synonym: *Phelipanche lavendulacea* (Rchb) Pomel, is a parasitic species with flower stalks averaging 15 to 60 cm high and 0.4 to 0.7 cm wide. *Orobanche lavandulacea* is a very rare taxon and has a steno-Mediterranean distribution, from Morocco and Spain on the west to Syria and Jordan, and along the Black Sea in the east (including also Canary Islands, France, Italy, Croatia, Greece, Cyprus, Algeria, Tunisia, Egypt, Turkey) [Sánchez Pedraja *et al*., 2005]. It appears to be a strict parasite of the Fabaceae family in the Mediterranean region [Mèdail *et al*., 2011]. *Orobanche lavandulacea* has been described in certain ethnicities as a medicinal plant with great qualities.

The biological activity of these species has scarcely been studied and little is known in terms of their phytochemistry.

FIGURE 1. Real images of *Cistanche violacea* collected in Dahar el Maztouria, *Orobanche crenata* and *Orobanche lavandulacea* collected in Dahar Cheneni, Tataouine, Tunisia. The distance between the two collection points is about 25 km. The flag on the map indicates the collection area.

The main reason may be related to the fact that these plants have mostly been recognized as weeds that cause agricultural issues [Prider *et al*., 2009] without prospecting for any economically profitable applications. Only a few investigations report the chemical and biological activities of these species [Bougandoura *et al*., 2016; Debouba *et al*., 2012]. The lipid profiles of the seeds of twenty-one *Orobanche* spp. (including *O. crenata* and *O. lavandulacea*) have been characterized [Velasco *et al*., 2000]. Phytochemical studies on different *Cistanche* spp. have isolated several chemical groups, including phenylethanoid glycosides, iridoids, and lignans (present in free and glycosidic forms), polysaccharides, free amino acids, ashes and minerals [Bougandoura, 2016; Deyama *et al*., 2006; Ebringerová *et al*., 1997; Wang *et al*., 2012]. Bougandoura *et al.* [2016] isolated a new iridoid and a new phenylethanoid glycoside with five known compounds from an aqueous-methanol extract of the aerial parts of *C. violacea*. The phenolic content, antioxidant power, and antimicrobial activities of *O. crenata* have been recently studied [Genovese *et al*., 2019], and its potential as a food prospect for human nutrition has also been shown [Renna *et al*., 2015].

In the present study, our goal was to extend the knowledge of three holoparasitic plants growing in the Dahar Natural region, in the southern of Tunisia (Figure 1), and to evaluate some aspects of their phytochemistry and antioxidant properties. Our findings can contribute to increase the perspectives for application of these plants in different areas such as in nutraceutical and functional food production.

MATERIALS AND METHODS

Chemicals and instrumentation

Deuterium oxide (D_2O, 99.9%), sodium 3-trimethylsilylpropionate-2,2,3,3,-d4 (TSP), 3-(4,5-dimethylthiazol-2-yl)--2,5-diphenyltetrazolium bromide (MTT) and standards of fatty acids and fatty acid methyl esters were purchased from Sigma–Aldrich (Milan, Italy). Methanolic HCl (3 N) was purchased from Supelco (Bellefonte, PA, USA). The cyanidin-3-*O*-glucoside (C3G) standard was acquired from Extrasynthese (Genay, France). Ultrapure water (18 mΩ) was obtained with a Milli-Q Advantage A10 System apparatus (Millipore, Milan, Italy). All other reagents, standards and solvents were of HPLC grade and used without further purification. All the spectrophotometric analyses were performed using UltroSpec 2100 pro (Amersham Bioscience, Milan, Italy).

Plant material

The plant species were harvested during the spring of 2017, from the region of Tataouine, south-eastern Tunisia. The species, all belonging to the family of Orobanchaceae and identified in the South Tunisian Flora, are: *C. violacea*, from the site named Dahar El Maztouria (DMM 32.853200, 10.446700), *O. crenata*, and *O. lavandulacea,* from Dahar Cheneni (DMM 32.918700, 10.268200, Figure 1). Plants were authenticated by Prof. Mohamed Chaieb based on the following flora: Pottier-Alapetite [1979, p. 1990], Flora Medcheklist (1984, 1986, 1989) [Greuter *et al*.], Dobignard & Chatelain [2010] and the Flora of Chaieb & Boukhris [1998]. A specimen of each collected species was deposited in the Laboratory of Botany at the University of Sfax, Tunisia, under number Tun. Flo. 39/2017. For each species, we collected the whole plant. During collection, samples were kept at approximately 4–8°C in a portable refrigerator (using dry ice to better control the temperature). Then, they were taken back to the laboratory where they were gently cleaned, and the residual soil was removed. The specimens (between 15 and 20 for each species) were cut into slices, approximately 0.5 cm thick, and freeze-dried (Telstar LyoQuest, Milan Italy). Samples were milled and prepared for the extraction step.

Preparation of extracts

Freeze-dried samples were extracted by cascade maceration using different solvents with increasing polarity. Briefly, 25 g of powder was mixed with 150 mL of *n*-hexane. After 24 h of incubation at 25°C in the dark, the supernatant was recovered by filtration. The organic solvent was removed using a rotary evaporator under vacuum (Rotavapor Buchi R200, Cornaredo, Italy). Then, the residues were recovered, and the process was repeated in cascade using ethyl acetate, acetone, methanol and water in turn as the solvent. After the last extraction process, water was removed using a freeze--dryer (Telstar LyoQuest). The dry extracts obtained were stored at -20°C. Three independent samples were extracted for each species to ensure statistical analysis of the results. Before each analysis, the extracts have been re-dissolved in the same solvents in stock solutions ranging 2–10 mg/mL, and then drastically diluted in water (or methanol) to ensure similar experimental conditions during the assays.

Oil extraction

Oil extractions were performed using a known amount of each plant. The powdered plant was transferred into a cellulose extraction thimble and inserted into a Soxhlet assembly fitted with a 100 mL flask. Fifty milliliters of *n*-hexane were added, and the whole assembly was heated for 6 h using a heating mantle. The extracts were concentrated using a rotary evaporator. The dry extracts obtained were stored at -20°C.

Determination of total phenolic content by the Folin--Ciocalteu method

Total phenolic content was determined using the Folin--Ciocalteu reagent [Slinkard & Singleton, 1977] by reference to the calibration curve obtained using gallic acid as a standard (linear range from 0.05 to 0.6 mM). The results were calculated in equivalents of gallic acid per gram of extract (mmol GAE/g).

Determination of flavonoids

The total flavonoid content contained in the extracts was estimated using a previously described method [Zucca *et al*., 2010]. Catechin was used for calibration (linearity range of 0.1–0.6 mM), and the results were calculated in catechin equivalents per gram of extract (mmol CE/g).

Determination of anthocyanins

The total content of anthocyanins was determined as previously described [Delazar *et al*., 2010; Zucca *et al*., 2016] according to the differential pH method. Five hundred μL

of extract was mixed with 0.5 mL of each buffer solution. The absorbances were measured at 510 nm and 700 nm with respect to the distilled water as a reference. Final absorbance was calculated using equation (1).

$$A = (A\,\lambda_{510} - A\,\lambda_{700})\ pH\ 1.0 - (A\,\lambda_{510} - A\,\lambda_{700})\ pH\ 4.5 \quad (1)$$

The molar extinction coefficient of C3G was used in the Lambert-Beer equation to calculate the anthocyanin content. It was expressed in mg equivalents of cyanidin-3-*O*--glucoside per gram of extract (mg C3G/g).

Measurement of antioxidant activity

DPPH radical scavenging activity

The antiradical activity was determined using the spectrophotometric method involving the deep purple 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [Brand-Williams *et al*., 1995]. A total of 700 μL of DPPH radical (25 mg/L) was mixed with 300 μL of each extract. The sample was mixed with a vortex for 30 s and then stood at room temperature for 30 min. The absorbance was measured at 515 nm. Trolox was used as a standard (linear range 5–50 μM), and the results were calculated in Trolox milliequivalents per gram of extract (mmol TE/g).

Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) assay was performed according to the Benzie & Strain [1996] method with some modifications. The test consisted of reacting 770 μL of water and 30 μL of each extract with 200 μL of the FRAP solution in glass hemolysis tubes. After 6 min of standing at room temperature and centrifugation for 10 min at 10,000 rpm, the absorbance of the supernatant was measured at 593 nm. FRAP was calculated in Fe(II) mmol per gram of extract (mmol Fe(II) / g).

Trolox Equivalent Antioxidant Capacity

The Trolox equivalent antioxidant capacity (TEAC) was determined using the spectrophotometric assay with a stable radical cation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) [Re *et al*., 1999]. Two solutions were prepared: a solution of ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (7 mM) and a solution of potassium peroxydisulfate (2.45 mM). ABTS radical cation was generated by mixing the two solutions in equivalent amounts. The mixture reacted for at least 12 h in the dark at room temperature. The obtained solution was diluted with 75 mM potassium phosphate buffer, pH 7.4, to a final absorbance of 0.70±0.05 measured at 734 nm. Then, 1 mL of $ABTS^{\bullet+}$ solution was mixed with 10 μL of extract solution and stood for 6 min. A decrease in absorbance at 734 nm was finally detected. Trolox was used as a standard, and the results were expressed in Trolox milliequivalents per gram of extract (mmol TE/g).

NMR SPECTROSCOPY

The aqueous extracts were dried under vacuum. A total of 10 mg of each dried extract was resuspended in 600 μL of D_2O containing the internal standard (TSP) added at a known concentration (1 mM) and vortexed. Then, 650 μL of the resulting solution was transferred to a 5 mm OD NMR tube.

^{1}H NMR analysis was performed on a spectrometer (Varian UNITY INOVA 500, Palo Alto, CA, USA) operating at 499.84 MHz. The spectra were acquired at 300 K from 256 scans collected over a spectral width of 6000 Hz. A 45° pulse, an acquisition time of 2.3 s and a relaxation time of 4 s were used. The removal of the water peak was obtained by applying a pre-saturation technique with low power radiofrequency irradiation for 1.5 s. A line broadening of 0.3 Hz was applied to all free induction decays (FIDs) prior to Fourier transformation. The phase and baseline of the spectra were manually corrected using MestReNova software (version 8.1.2, Mestrelab Research SL, Santiago de Compostela, Spain), and ^{1}H NMR chemical shifts were referred to the TSP resonance at δ 0.00 ppm.

The molecular composition of the extracts, expressed in mol%, was estimated by measuring the area under the NMR peaks assigned to the specific functional groups of molecules and normalizing the integrals to the sum of the total spectra to compensate for possible differences in the extraction yields. The MHz library in Chenomx NMR suite version 7.5 (evaluation edition, Chenomx Inc., Edmonton, AB, Canada) was used for data interpretation.

Fatty acid analysis

The fixed oils of the plant species (3 mg, in EtOH solution) obtained by oil extraction were subjected to mild saponification as previously reported [Rosa *et al*., 2012]. Dried saponifiable fractions were dissolved in acetonitrile with 0.14% (v/v) acetic acid and then analyzed by high-performance liquid chromatography (HPLC-DAD). A portion of dried fatty acids after saponification was methylated with 3N methanolic HCl (at room temperature) as reported, and fatty acid methyl esters (FAME) were analyzed by gas chromatography (GC-FID). All solvents were evaporated under vacuum.

FAME were measured on a gas chromatograph HP--6890 (Hewlett-Packard, Palo Alto, CA, USA) with a flame ionization detector (GC-FID) and equipped with a cyanopropyl methyl-polysiloxane HP-23 FAME column. FAME were identified with standard compounds and quantified as a percentage of the total fatty acids (FA). The unsaturated fatty acids (UFA) composition of plant oils was obtained with an 1100 HPLC-DAD system (Agilent Technologies, Palo Alto, CA, USA). UFA (detected at 200 nm) were eluted with $CH_3CN/H_2O/CH_3COOH$ (75/25/0.12, *v/v/v*) as the mobile phase at a flow rate of 2.3 mL/min using an Agilent Technologies XDB-C_{18} Eclipse column. UFA were identified using standard compounds (linear calibration curves, correlation coefficients > 0.995) and conventional UV spectra; chromatogram data were recorded and integrated with an Agilent OpenLAB Chromatography data system.

Tumor cell cultures

The B16F10 cell line from mouse melanoma was obtained from the Interlab Cell Line Collection (ICLC) (IRCCS Az. Osp. Univ. San Martino, Genova, Italy). Sub-

TABLE 1. Phenolic composition and antioxidant activity of the *C. violacea*, *O. crenata*, and *O. lavandulacea* extracts (data are expressed per gram of dry extract).

	Plant name	Hexane	Ethyl acetate	Acetone	Methanol	Water
Extraction yield (g/100 g DM)	*C. violacea*	1.4±0.2	1.0±0.3	1.7±0.6	26.4±0.7	27.3±1.1
	O. crenata	1.3±0.4	0.5±0.1	2.4±0.5	11.8±0.2	18.5±0.6
	O. lavandulacea	1.1±0.1	0.5±0.1	3.8±0.4	5.5±0.3	15.8±0.3
DPPH• scavenging activity (mmol TE/g)	*C. violacea*	2.44 ± 0.02^{d}	1.14 ± 0.01^{g}	7.16 ± 0.17^{a}	0.89 ± 0.01^{gh}	1.10 ± 0.008^{g}
	O. crenata	0.64 ± 0.01^{hi}	2.40 ± 0.17^{d}	5.30 ± 0.02^{c}	0.50 ± 0.06^{i}	0.33 ± 0.006^{i}
	O. lavandulacea	2.00 ± 0.03^{e}	6.80 ± 0.35^{b}	1.61 ± 0.05^{f}	1.43 ± 0.01^{fg}	0.30 ± 0.003^{i}
TEAC (mmol TE/g)	*C. violacea*	15.71 ± 0.2^{d}	6.35 ± 0.1^{efg}	42.01 ± 0.6^{a}	13.01 ± 0.01^{d}	12.70 ± 0.03^{d}
	O. crenata	4.70 ± 0.1^{gh}	20.06 ± 0.4^{c}	14.14 ± 0.2^{d}	3.34 ± 0.09^{gh}	1.52 ± 0.02^{h}
	O. lavandulacea	8.65 ± 1.0^{ef}	23.71 ± 1.5^{b}	8.57 ± 1.0^{ef}	9.08 ± 0.80^{ef}	5.67 ± 0.30^{fg}
FRAP (mmol Fe(II)/g)	*C. violacea*	1.26 ± 0.02^{fg}	1.26 ± 0.04^{fg}	4.84 ± 0.10^{b}	0.20 ± 0.09^{j}	0.19 ± 0.006^{j}
	O. crenata	0.40 ± 0.02^{i}	28.67 ± 0.07^{a}	3.24 ± 0.06^{c}	0.73 ± 0.01^{h}	0.44 ± 0.008^{i}
	O. lavandulacea	1.09 ± 0.03^{g}	1.31 ± 0.04^{ef}	1.53 ± 0.02^{d}	1.49 ± 0.02^{de}	0.32 ± 0.006^{ij}
Total phenolic content (mmol GAE/g)	*C. violacea*	0.60 ± 0.09^{f}	1.0 ± 0.02^{d}	1.50 ± 0.04^{c}	0.68 ± 0.04^{ef}	0.69 ± 0.07^{ef}
	O. crenata	0.50 ± 0.01^{f}	1.71 ± 0.03^{c}	3.53 ± 0.20^{a}	2.12 ± 0.01^{b}	1.43 ± 0.09^{c}
	O. lavandulacea	0.52 ± 0.01^{f}	2.07 ± 0.06^{b}	0.93 ± 0.02^{de}	2.29 ± 0.02^{b}	1.09 ± 0.01^{d}
Total flavonoid content (mmol CE/g)	*C. violacea*	0.07 ± 0.010^{i}	0.79 ± 0.03^{de}	0.80 ± 0.08^{de}	0.23 ± 0.01^{g}	0.47 ± 0.04^{f}
	O. crenata	0.02 ± 0.001^{j}	6.78 ± 0.20^{a}	0.75 ± 0.10^{de}	0.45 ± 0.03^{f}	0.64 ± 0.05^{ef}
	O. lavandulacea	0.15 ± 0.009^{h}	4.53 ± 0.20^{b}	0.37 ± 0.04^{f}	2.39 ± 0.08^{c}	0.92 ± 0.02^{d}
Total anthocyanin content (mg C3G/g)	*C. violacea*	6.6 ± 0.4^{bc}	5.3 ± 0.5^{c}	21.6 ± 1.2^{a}	0.27 ± 0.01^{g}	0.70 ± 0.03^{f}
	O. crenata	4.7 ± 0.2^{cd}	22.4 ± 0.3^{a}	4.3 ± 0.3^{d}	8.36 ± 0.40^{b}	3.22 ± 0.07^{e}
	O. lavandulacea	3.7 ± 0.2^{de}	21.2 ± 0.4^{a}	3.6 ± 0.3^{de}	4.7 ± 0.10^{cd}	5.26 ± 0.10^{c}

Data are expressed as mean ± SD (n =3). Mean values for the same analysis having different letters are significantly different ($p<0.05$; One–way ANOVA followed by the Bonferroni Multiple Comparisons Test). DM: dry material. TEAC: Trolox equivalent antioxidant capacity. FRAP: Ferric reducing antioxidant capacity. GAE: Gallic acid equivalents. CE: Catechin equivalents. C3G: cyanidin-3-*O*-glucoside.

cultures of B16F10 cells were cultured in T-75 culture flasks and passed with a trypsin-EDTA solution. The cells were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL) (Invitrogen) in a 5% CO_2 incubator at 37°C.

MTT test of cell viability

The cytotoxic effect of oils was evaluated in cancer B16F10 cells by the MTT assay. Cancer B16F10 cells, cultured according to Rosa *et al.* [2015], were exposed to different aliquots of oils (50–500 μg/mL, from a 20 mg/mL solution in EtOH) in complete culture medium for 24 h. Treated cells were compared to untreated cells (control cells, receiving no treatment) and vehicle-treated cells (incubated for 24 h with an equivalent volume of EtOH; maximal final concentration, 1.2%) for viability. After incubation, B16F10 cells were subjected to the MTT assay as previously reported. The color change was measured at 570 nm with a microplate reader (Infinite 200, Tecan, Mannedorf, Switzerland); the absorbance is proportional to the number of viable cells.

Statistical analysis

GraFit 7 (Erithacus Software, London, United Kingdom), R 2.5.1 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad INSTAT (GraphPad Software, San Diego, CA, USA) were used for data analysis. The Pearson correlation coefficient (r) was calculated using GraphPad INSTAT. One-way analysis of variance (ANOVA) and the Bonferroni multiple comparison test were used to assess the statistical significance of the differences. All the analyses were performed at least in triplicate (unless otherwise stated), and the data are reported as mean ± standard error of the mean (SEM) or standard deviation (SD).

RESULTS AND DISCUSSION

As it could be seen in Table 1, the yield of the different extractions varied considerably depending on the plant and the solvent used. *C. violacea* gave the best yield for the aqueous and methanolic extracts, with 27.3% and 26.4%, respectively. The lowest yield was obtained for both *Orobanche* ethyl acetate extracts (0.5%). These data suggest that the majority of the compounds extracted present high polarity.

Quantification of total phenolics, flavonoids and anthocyanins, and antioxidant activity

Total phenolics content of the extracts varied greatly (from 0.50 mmol GAE/g for the *O. crenata* hexane extract to 3.53 mmol GAE/g for the *O. crenata* acetone extract). The two *Orobanche* species were richer in phenolics compared to the *Cistanche* species. However, the total phenolic contents of *O. crenata* and *O. lavandulacea* extracts were quite different. The acetone extract of *O. crenata* was the richest in phenolics, whereas the *O. lavandulacea* showed higher (or similar) total phenolic content for the ethyl acetate and methanol extracts.

As flavonoids constitute a significant portion of phenolics in *Cistanche* spp. [Debouba, 2012], they have also been quantified in our samples. The highest total flavonoid content was observed in the ethyl acetate extracts of *O. crenata* and *O. lavandulacea* with 6.78 and 4.53 mmol CE/g, respectively (both statistically higher than the values determined for the other extracts at $p<0.05$).

Among flavonoids and their derivatives, anthocyanins are colored compounds (orange, purple to blue). Although widely involved in the coloring of petals, they are also found in many tissues. Their synthesis in foliar organs is often activated by stress (cold, deficiencies, senescence, *etc*.). All studied parasite species were well colored and exhibited significant amounts of anthocyanins. Thus, the highest total anthocyanin contents were present in the ethyl acetate extract of *O. crenata* (22.43 mg C3G /g), acetone extracts of *C. violacea,* and ethyl acetate extract of *O. lavandulacea* (21.59 and 21.17 mg C3G/g, respectively, $p<0.05$ in comparison with all the other data). In this case, a different distribution of compounds among the solvents could be seen, possibly suggesting a different anthocyanin composition among the three studied species. Such data need however confirmation by more precise chromatographic quantifications.

Several *in vitro* methods have been recommended to evaluate the antioxidant activity of plant extracts [Schlesier *et al.*, 2002; Zucca *et al.*, 2013]. There are, in fact, many methods differing in their analytical principles and experimental conditions, and antioxidant molecules give different contributions to the total antioxidant potential [Craft *et al*., 2012]. In the present study, we used DPPH, ABTS (TEAC), and FRAP assays to evaluate the *in vitro* antioxidant activity of *C. violacea*, *O. crenata*, and *O. lavandulacea* extracts (Table 1). The extracts with the highest free radical scavenging activity ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) also showed the highest reducing ability of Fe(III) (FRAP). This probably reflects the presence of antioxidant molecules in our extracts that can intervene by two types of reaction mechanisms [Craft et al., 2012]. For the FRAP, the reduction of Fe(III) is based exclusively on an electron transfer. Regarding the DPPH and ABTS assays, these radicals can, in fact, be neutralized either by direct reduction *via* electron transfers or by radical scavenging *via* a transfer of a hydrogen atom. The present study shows that all the plants investigated have antioxidant activities that vary considerably from one species to another. The highest antioxidant activity, based on all the tests combined, was obtained with the *C. violacea* species, followed by the two *Orobanche* species.

The highest TEAC was recorded for the following extracts: the acetone extract of *C. violacea* with 42.01 mmol TE/g, the ethyl acetate extract of *O. lavandulacea* with 23.71 mmol TE/g, and the ethyl acetate extract of *O. crenata* with 20.06 mmol TE/g. The last mentioned extract also had the highest FRAP (28.67 mmol TE/g). The DPPH assay showed lower values than TEAC; this could be probably due to some different affinity to the two radicals among the chemicals of the extracts.

TABLE 2. Correlation coefficient (r) calculated between the content of phenolic groups and the results antioxidant assays.

	DPPH	TEAC	FRAP
C. violacea			
Total phenolics	0.8445	0.7823	0.9187*
Total flavonoids	0.4356	0.3470	0.5714
Total anthocyanin	0.9791*	0.9104*	0.9975*
O. crenata			
Total phenolics	0.8155	0.4101	0.0116
Total flavonoids	0.2025	0.8118	0.9964*
Total anthocyanin	0.0910	0.7448	0.9623*
O. lavandulacea			
Total phenolics	0.4492	0.5345	0.3584
Total flavonoids	0.8210	0.8765	0.2560
Total anthocyanin	0.9439*	0.9660*	0.1246

* statistical significant ($P<0.05$).

The results (Table 2) showed that the antioxidant capacity evaluated through all these methods correlated with the contents of phenolic compounds, confirming their predominant role in this context. The highest correlation coefficient values were more frequently obtained for correlation between results of antioxidant assays and total anthocyanin contents, thus suggesting their predominant contribution in the antioxidant capacity of the samples.

Most of the published studies on the plant species selected for this study is limited to the assessment of the antioxidant capacity of essential oils. Very little has been reported in the literature on the antioxidant activity of non-volatile extracts in the plant. Particularly, we found little reports on the total phenolic content of *C. violacea* and its antioxidant activity [Bouchouka, 2016]. This species, harvested in Tunisia, was the subject of a single study on the antioxidant capacity of the aqueous and methanolic extract with the DPPH assay whose IC_{50} values were 6.38 and 17.15 μg/mL for the flowers and the bulb, respectively [Debouba *et al*., 2012]. The same study showed that the flowers had the strongest iron reducing and the bulb had the strongest chelating effect. Concerning the *C. violacea* that grows in Algeria, only one recent investigation has focused, for the first time, on the anti-inflammatory activity of a few isolated chemical constituents of the ethyl acetate fraction of the aqueous/methanolic extract of aerial parts of the plant [Bougandoura *et al*., 2016]. Regarding the two *Orobanche*, to the best of our knowledge, there is no published investigation on the phytochemical content and antioxidant activity of *O. lavandulacea*, whereas the study

of Nada & El-Chaghaby [2015] showed that *O. crenata* harvested from Egypt could be a potential source of nutrients, antioxidants, and antibacterial compounds.

On the other hand, in the present work, we also found a good positive correlation between the results of three assays used to evaluate the antioxidant capacity. This presumably indicates that in an extract, the phenolics provide the radical scavenging activity ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) and possibly are themselves responsible for the ability to reduce ferric ions. Some authors report that the antioxidant capacity of natural substances could have a reciprocal correlation with their reducing capacity [Guettaf *et al*., 2016] and that the latter can, in turn, serve as an important indicator of their potential for antioxidant activity [Wang *et al.*, 2012].

^{1}H NMR spectroscopy

The chemical profiles of the aqueous extracts of the three selected species were characterized by ^{1}H NMR spectroscopy. Representative spectra are shown in Figure 2. Spectral resonances of metabolites were attributed by using literature data [HMDB, 2019]. The high field region of the spectra (0–3.0 ppm) revealed the presence of amino acids such as alanine, asparagine, and valine and organic acids including acetate, citrate, malate, malonate, and succinate. The middle spectral region of all extracts (3.0–5.5 ppm) was dominated by the signals from carbohydrates (mannitol, glucose, fructose, sucrose, and fucose). In addition, betaine and choline were also identified in this portion of the spectrum. The low field region of the spectra (5.5–9 ppm) showed the weakest signals, mainly from aromatic compounds.

The relative abundances of 16 metabolites (five carbohydrates, six organic acids, three amino acids, and two osmoprotectants) were determined by considering easily integrated peaks. The results are shown as mol% in Table 3. Inspection of the table shows that mannitol was the most abundant carbohydrate in all the samples. Concerning the class of organic acids, all extracts were particularly rich in malic acid, especially *O. lavandulacea*. Higher levels of acetic acid were found in *O. crenata* and *O. lavandulacea* than in *C. violacea*. The accumulation of asparagine was significantly higher in *C. violacea* than in *O. lavandulacea*, while the presence of this amino acid was not revealed in the extract of *O. crenata*. Finally, the betaine content was much higher in the *C. violacea* extract than in the two *Orobanche* species.

It is known that the content of soluble sugars and other carbohydrates, free amino acids, organic acids and osmoprotectants in plants may act as metabolic signals in response to several environmental factors, such as drought, salinity and nutrient imbalances. Tolerance to abiotic stresses is very complex due to both the complexity of interactions between stress factors and various molecular, biochemical and physiological phenomena affecting plant growth and development. Although the presented NMR results are not enough to compare the metabolism of the three plants under investigation, the presence of the abovementioned differences in composition is a clear indication of different biological adaptabilities to the harsh environment of the Dahar region.

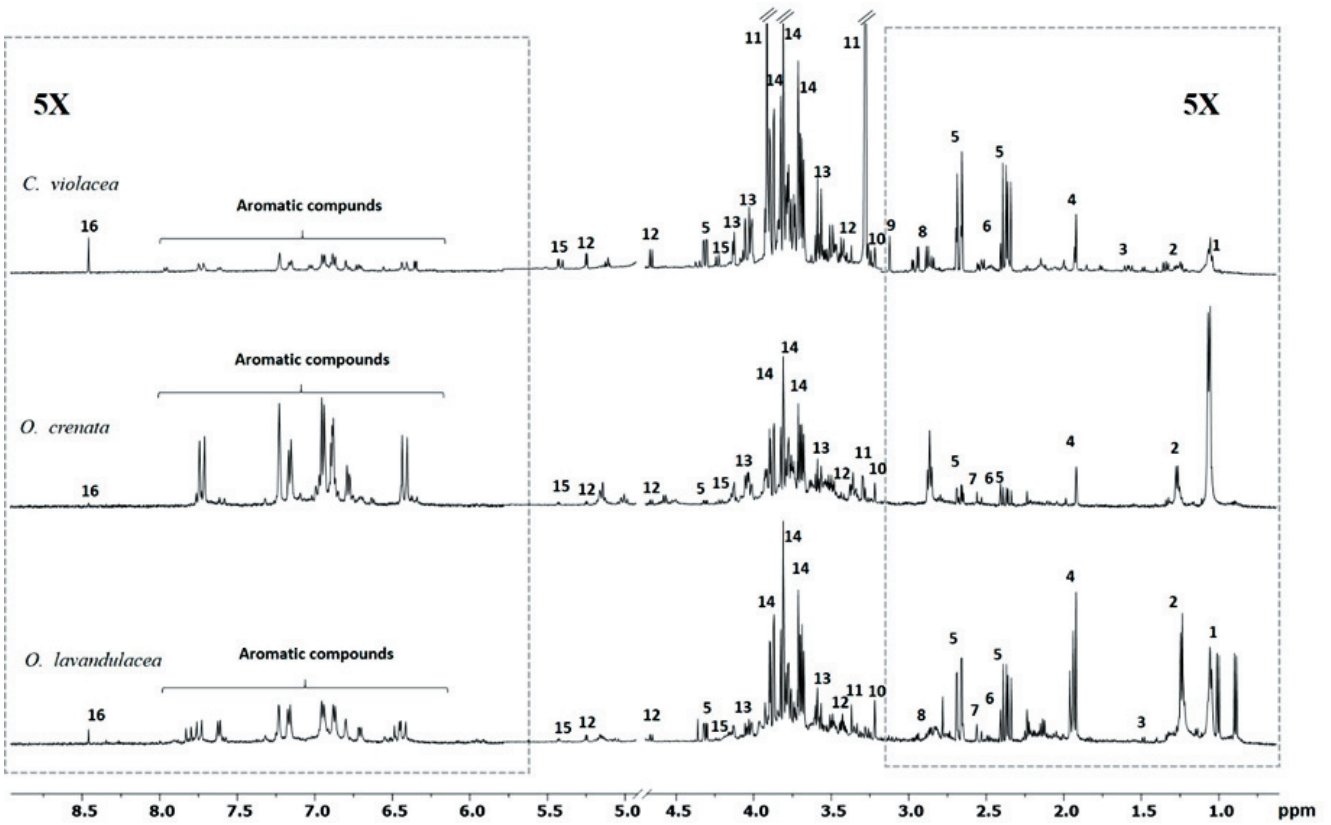

FIGURE 2. Stack-plot of ^{1}H NMR spectra of the aqueous extracts of *C. violacea*, *O. crenata,* and *O. lavandulacea*. Key: (1) valine, (2) fucose, (3) alanine, (4) acetate, (5) malate, (6) succinate, (7) citrate, (8) asparagine, (9) malonate, (10) choline, (11) betaine, (12) glucose, (13) fructose, (14) mannitol, (15) sucrose, (16) formate.

TABLE 3. Composition (mol %) of the aqueous extracts of *C. violacea*, *O. crenata* and *O. lavandulacea* estimated by the analysis of the corresponding ^{1}H NMR spectra.

Metabolite	*Cistanche violacea*	*Orobanche crenata*	*Orobanche lavandulacea*
Carbohydrates			
Glucose	8.22±0.43^a	7.58±1.04^a	7.25±2.30^a
Fructose	14.72±1.67^b	30.43±1.96^a	15.35±1.12^b
Sucrose	2.06±0.35^a	2.16±0.32^a	1.40±0.09^b
Mannitol	16.81±2.86^c	39.99±2.62^a	34.87±1.00^b
Fucose	0.27±0.02^b	1.10±0.11^a	1.32±0.46^a
Amino acids			
Alanine	0.14±0.02^b	nd	0.60±0.12^a
Asparagine	2.64±0.84^a	nd	1.31±0.72^b
Valine	0.08±0.01^b	nd	0.46±0.21^a
Organic acids			
Acetate	0.62±0.18^b	2.40±0.55^a	3.50±1.14^a
Citrate	nd	1.49±0.09^a	1.52±0.31^a
Formate	0.69±0.10^a	0.67±0.07^a	1.12±0.68^a
Malate	15.68±0.71^b	9.70±0.75^c	25.46±5.09^a
Malonate	0.96±0.01	nd	nd
Succinate	0.23±0.01^b	0.69±0.04^a	0.51±0.17^a
Other compounds			
Betaine	36.59±0.17^a	0.67±0.04^b	0.38±0.01^c
Choline	0.28±0.01^b	0.87±0.13^a	0.84±0.08^a

Data are expressed as mean values ± standard deviations (SD), n=3; nd.: not detectable; Mean values in the same row having different letters are significantly different (P<0.05; One–way ANOVA followed by the Bonferroni Multiple Comparisons Test).

TABLE 4. Fatty acid composition (% of total fatty acids) obtained by GC--FID analysis of *C. violacea* (CvO), *O. crenata* (OcO) and *O. lavandulacea* (OlO) SE oils.

Fatty acid	CvO	OlO	OcO
12:0	9.49±0.35^a	0.57±0.03^c	1.92±0.21^b
14:0	7.98±0.72^a	4.36±0.04^b	8.41±1.09^a
16:0	28.66±1.60^b	33.36±1.13^a	32.07±1.26^a
16:1 *n-7*	1.89±0.40^b	2.48±0.09^a	2.79±0.12^a
18:0	4.37±0.58^a	4.09±0.86^a	3.36±0.93^a
18:1 *n-7*	1.01±0.3^b	1.67±0.04^a	1.20±0.32^{ab}
18:1 *n-9*	9.71±0.88^a	9.63±0.13^a	6.88±0.71^b
18:2 *n-6*	30.78±1.08^b	23.95±0.32^c	34.36±1.47^a
18:3 *n-3*	0.33±0.36^c	18.92±0.25^a	8.29±0.77^b
SFA	50.49±3.13^a	42.69±0.62^b	46.04±2.66^{ab}
MUFA	12.61±1.29^{ab}	13.79±0.07^a	10.86±0.93^b
PUFA	34.11±1.44^b	42.87±0.29^a	42.66±2.23^a

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. All data are expressed as mean values ± standard deviations (SD); (n = 4). Mean values in the same row having different letters are significantly different (P<0.05; One–way ANOVA followed by the Bonferroni Multiple Comparisons Test).

Fatty acid profiles of parasitic plant oils

The GC-FID and HPLC-DAD analyses allowed us to obtain qualitative information on the individual FA composing the three oil extracts: *C. violacea* (CvO), *O. crenata* (OcO), and *O. lavandulacea* (OlO). Table 4 shows the composition of FA (expressed as % of total FA, g/100 g) of the oils obtained by GC-FID analysis. The content of saturated FA (SFA) in CvO was approximately 50%, which essentially contained lauric acid 12:0, myristic acid 14:0, palmitic acid 16:0, and stearic acid 18:0, with contents of 9.49%, 7.98%, 28.66%, and 4.37%, respectively. The CvO content of monounsaturated FA (MUFA) was approximately 12.61%, which was mainly composed of oleic acid 18:1 *n*-9 (9.71%) and palmitoleic acid 16:1 *n*-7 (1.89%), while the amount of polyunsaturated FA (PUFA) was 34.11%, which was mainly represented by the essential FA linoleic acid (18:2 *n*-6) and α-linolenic acid (18:3 *n*-3) (3.78% and 0.33%, respectively).

The absolute content of the main FA of CvO was detected by HPLC and the results were as follows: 118.5 mg/g, 41.4 mg/g, and 15.2 mg/g of 18:2 *n*-6, 18:1 *n*-9, and 18:3 *n*-3, respectively (Table 5). The two *Orobanche* oils showed different FA profiles compared to CvO, while some differences were observed between them. OlO showed a concentration of approximately 42.69% SFA, mainly 16:0 (33.36%), 14:0 and 18:0 (about 4% each); 13.79% MUFA, mainly 18:1 *n*-9 (9.63%); and 43% PUFA, mainly 18:2 *n*-6 (23.95%) and 18:3 *n*-3 (18.92%). OcO, compared to OlO, showed higher values of SFA (46.04%, with 32.07% of 16:0 and 8.41% of 14:0), a lower amount of MUFA (10.86%, with 6.88% of 18:1 *n*-9), and equal values of PUFA (42.66%), mainly represented by 18:2 *n*-6 (34.36%) and 18:3 *n*-3 (8.29%). The amounts of the main UFA in OlO and OcO determined by HPLC analysis were 172.0 and 201.0 mg/g for 18:2 *n*-6, 143.9 and 54.4 mg/g for 18:3 *n*-3, 81.2 and 47.4 mg/g for 18:1 *n*-9, respectively. All species exhibited a high level of SFA and PUFA. The main fatty acids were linoleic, palmitic and oleic acids in CvO; palmitic, linoleic and α-linolenic acids in OlO; and linoleic, palmitic and lauric acids in OcO. Significant differences were observed among oils in the levels of *n*-3 (ω-3) FA; in particular, OlO emerged as the richest in 18:3 *n*-3 (p<0.05). Moreover, all oils showed high levels of essential FA, which represented 31.1%, 42.9%, and 42.6% of the total FA content, in CvO, OlO, and OcO, respectively. The differences observed in the FA profiles of the oil could be attributable to several factors, such as variances in plant lipid metabolism, the impact of the harvest location (availability of climate, soil and water), and the diversity of metabolic precursors derived from different host plants.

Lipid content and composition are essential factors if a plant is to be considered from a food point of view. High levels of oleic (55–61%), linoleic (27–38%), and palmitic (6–9%)

TABLE 5. Unsaturated fatty acid composition (mg/g of oil) obtained by HPLC-DAD analysis of *C. violacea* (CvO), *O. crenata* (OcO), and *O. lavandulacea* (OlO) oils.

Fatty acid	CvO	OlO	OcO
16:1 *n*-7	2.43±0.54^{c}	8.62±0.62^{b}	10.07±0.24^{a}
18:1 *n*-9	41.44±0.28^{c}	81.19±4.28^{a}	47.40±0.32^{b}
18:2 *n*-6	118.47±2.69^{c}	172.02±3.59^{b}	201.00±1.83^{a}
18:3 *n*-3	15.23±0.46^{c}	143.86±3.29^{a}	45.44±0.56^{b}

All data are expressed as mean values ± standard deviations (SD); (n = 4). Mean values in the same row having different letters are significantly different (P<0.05; One–way ANOVA followed by the Bonferroni Multiple Comparisons Test).

acids have been previously reported in the seeds of *O. crenata* and *O. lavandulacea* [Velasco *et al*., 2000]. In contrast, our result showed that the lipids extracted from the whole plants contained predominantly linoleic and palmitic acids, since the pathways of lipid synthesis/accumulation may vary depending on the tissue [Chapman *et al*., 2012]. Moreover, OlO emerged as the richest in 18:3 *n*-3, an essential fatty acid that has been reported to modulate inflammatory responses in animal models and clinical trials and to reduce the cardiovascular disease [Rosa *et al.*, 2012].

The FA composition of oils makes these parasitic plants suitable for nutritional application because they provide a rich source of functional compounds (essential FA) with potential health benefits.

Cytotoxicity on B16F10 mouse melanoma cells

There is a great interest in the potential anticancer properties of unconventional vegetable oils and several reports have described interesting antimutagenic activities of fixed oils obtained from plants and herbs used in traditional medicine [Rosa *et al*., 2012]. Moreover, the oil extracted from the edible parasitic plant *Cynomorium coccineum* showed a significant cytotoxic effect on melanoma and colon cancer cells. Therefore, in the present study, we tested the cytotoxic effect of oils from the three parasitic species by MTT assay in B16F10 mouse melanoma cells, a metastatic cancer tyrosinase-expressing cell line [Casañola-Martin *et al*., 2014] used to screen natural antitumor extracts and lipid compounds. Figure 3 shows the viability, expressed as % of the control, measured in B16F10 cells after 24 h of incubation in the presence of different concentrations (50–500 μg/mL) of oil extracts. OlO showed the highest cytotoxic effect with respect to CvO and OcO. The treatment with OlO induced a significant reduction in cell viability at a concentration of 50 μg/mL in comparison with the control, with a viability reduction of 95% at 500 μg/mL. The treatment with OcO induced a significant reduction (P<0.001) in cell viability (45%) at only 500 μg/mL. EtOH, used to dissolve oils, was not toxic in cancer cells, and cell viability, measured at the maximal tested dose (1.2%), was 97%. CvO exhibited a significant cytotoxic effect from the 250 μg/mL dose, causing a viability reduction of 14% at the highest tested doses.

On the whole, our results showed that all the plant oils used in this study have a certain cytotoxic activity, which is manifested by the decrease in the viability of the B16F10 cells. None of the parasitic plants belonging to the Orobanchaceae family was previously used for viability tests on B16F10. Only one group reported a certain cytotoxicity of 3'-*O*-methyl isocrenatoside and methyl caffeate extracted from *Orobanche cernua* [Qu *et al*., 2016].

Among three plant species studied, *O. lavandulacea* was revealed to be a highly cytotoxic species for B16F10. In this

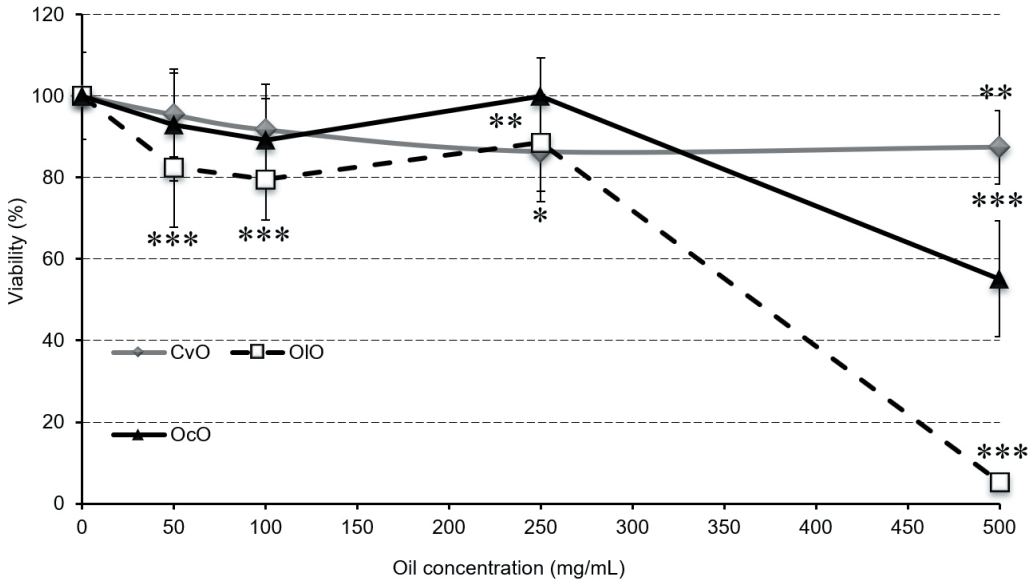

FIGURE 3. Viability, expressed as % of the control, induced by 24 h of incubation with different concentrations (50–500 μg/mL) of oils obtained from *C. violacea* (CvO), *O. crenata* (OcO), and *O. lavandulacea* (OlO) in B16F10 cancer melanoma cells (MTT assay). Three independent experiments were performed, and the data are presented as the mean ± SD; *** P<0.001; ** P<0.01; * P<0.05 *versus* control; (n=16) (one–way ANOVA followed by the Bonferroni Multiple Comparisons Test).

study, OlO emerged as the richest in 18:3 *n*-3 and several plant-based oils rich in α-linolenic acid, such as canola and flax seed oils, have shown the ability to modulate cancer cell growth and death. In particular, the fixed oil obtained from the aerial part of the parasitic plant *C. coccineum* showed a significant growth inhibitory effect on melanoma and colon cancer cells and its antitumor activity has been related to the oil level of 18:3 *n*-3 and the ability of this essential fatty acid to be the precursor in cancer cells for the formation of long chain *n*-3 PUFA (EPA and DHA) with anticancer activity [Rosa *et al*., 2012, 2015]. Nevertheless, further studies are needed to identify the OlO compounds responsible for the observed activity.

CONCLUSIONS

On the whole, these results highlighted the antioxidant activity measured as DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activity and the ability to reduce ferric ions, as well as cytotoxic activity on B16F10 cells of three plants of Orobanchaceae family (*C. violacea*, *O. crenata*, and *O. lavandulacea*). Our findings support the role and value of wild foods in agricultural systems, despite the harsh climate conditions of growth. Despite the lack of a complete phytochemical characterization (one of the future required steps), such results could help in the valorization of these plants, contributing in preserving biodiversity. The reported observations, in fact, open the path to other lines of research, with the aim of improving the value of these species, and applications of these plants in several perspectives such as in nutraceutical and food production, in accordance with a possible use of parasitic plants as good alternative for expensive vegetables.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

RESEARCH FUNDING

This research was partially funded by grants from Sfax University, Tunisian Ministry of Higher Education and Scientific Research, University of Cagliari (F.I.R. 2017–2018) and Italian Ministry of University and Research (F.F.A.B.R. 2018).

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Submitted: 1 November 2019. Revised: 26 February, 21 March, 14 April, and 21 April 2020. Accepted: 5 May 2020. Published on-line: 9 June 2020.