Total Phenolic Contents, Antioxidant Activities and Cytotoxicity of Three *Centaurea* Species: *C. calcitrapa* subsp. *calcitrapa*, *C. ptosimopappa* and *C. spicata*

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ABSTRACT

Background: Many species of the genus *Centaurea* have been reported to be used in Anatolian folk medicine. In the present study, methanolic and aqueous extracts of three species of *Centaurea L.* (*C. calcitrapa* subsp. *calcitrapa*, *C. ptosimopappa* and *C. spicata*) have been assessed for antioxidant and cytotoxic activities. **Materials and Methods:** Antioxidant activity was investigated by DPPH and superoxide anion scavenging and anti-lipid peroxidation assays. Cytotoxic effect on HeLa and Vero cells were investigated by MTT assay. **Results:** Superoxide anion scavenging activities of aqueous extracts were higher than those of methanolic extracts, and the highest activity was detected in the aqueous extracts of *C. calcitrapa* subsp. *calcitrapa* with the IC_{50} value of 25.91 µg/mL. The methanolic extract of *C. ptosimopappa* showed the strongest anti-lipid peroxidation activity. Total phenolic contents (mg gallic acid equivalent per gram) were ranged between 17.25 and 120.50 and the highest value was detected in aqueous extract of *C. ptosimopappa*. The highest cytotoxic activity was observed in the methanolic extract of *C. calcitrapa* subsp. *calcitrapa* on both cell lines with the IC_{50} values of <100 µg/mL. **Conclusions:** According to the results of activity tests, the selected *Centaurea* species, especially *C. calcitrapa* subsp. *calcitrapa* can be regarded as a potential source of natural antioxidants in the prevention and treatment of diseases in which oxidants or free radicals are implicated.

Key-words: Antioxidant activity; *Centaurea*; Cytotoxicity; Free radical scavenging.

Key Messages: The selected *Centaurea* species showed an antioxidant activity and very low cytotoxic activity. Thus, *Centaurea* species might be a good candidate for further studies targeting the development of new antioxidants.

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INTRODUCTION

Medicinal plants have long been used for treatment of various diseases. In Turkey, approximately 9000 species are employed commonly in the practice of traditional medicine.[1] However, therapeutic importance of many medicinal plants has not been recognized yet.

The genus *Centaurea* L. is the third largest genus in Turkey.[2,3] This herb is known with its Turkish names such as "kotankıran", "peygamberçiçeği", "çobankaldıran", "acımık", "sarıbaş", "kötürüm", and "çobandikeni".[4] Many species of this genus are traditionally used for the treatment of various ailments,[5] especially in Anatolian folk medicine.[4,6]

Members of *Centaurea* species were proposed to have antimicrobial,[5,7-10] antifungal,[8,11] anti-inflammatory,[12] anti-ulcerogenic,[13] antioxidant,[14-17] antiplasmodial,[18] antiprotozoal,[8] antiviral[19] and cytotoxic[18,20-22] activities.

C. calcitrapa which grows up on northwestern Anatolia is used (2-6% infusion) as a fever reducer. The ethanol: water (4:1) extract of *C. calcitrapa* was shown to have strong antioxidant activity.[14] *C. spicata* distributed around Amanos Mountains, Hatay-Mersin in Turkey. *C. ptosimopappa* is an endemic species and widespread

in the Amanus and Cassius mountains.[2,23] Previous investigations on various extracts and oil allowed the identification of nine guaiane-type sesquiterpene lactones, sesquiterpenes and two butyrolactone lignans in *C. ptosimopappa*, and the oil has been reported to possess antimicrobial activity.[7,10,24]

However, there is no information about antioxidant and cytotoxic activity of methanolic and aqueous extracts of *Centaurea calcitrapa* subsp. *calcitrapa, C. spicata* and *C. ptosimopappa*. The aim of the present study was to evaluate the *in vitro* antioxidant and cytotoxic activities of aqueous and methanolic extracts of these three *Centaurea* L. species, and to correlate the results with total phenolic contents.

MATERIALS AND METHODS

Plant Materials: Localities and collection periods of the species studied are as follows:

1. *C. calcitrapa* subsp. *calcitrapa* (Cc): Arsuz, İskenderun, Hatay, Turkey; July, 2003
2. *C. spicata* (Cs): Hatay: Kuyuluk Village, Amanos Mountains, Erzin, Hatay, Turkey; July, 2003
3. *C. ptosimopappa* (Cp): Dörtyol, Amanos Mountains, Hatay, Turkey; June, 2003

The voucher specimens were deposited at the Herbarium of the Department of Biology, Istanbul University, Istanbul, Turkey (Voucher No: 1, ISTF 40036; Voucher No: 2, ISTF 40029; Voucher No: 3, ISTF 40063, respectively).

Preparation of Extracts: For preparation of crude extracts, 20 g air-dried and powdered leaves of each plant were extracted with distilled water or methanol (20 mL/g) at room temparature, the process was repeated three times. Plant material was discarded by filtration and clarified extract was evaporated under vacuum to dryness using a rotary evaporator. The crude extracts were stored in dark at -20 ºC after determination of dry weight.

Total Phenolic Content (TPC): TPC was measured using the Folin-Ciocalteu's reagent as described by Ragazzi and Veronese.[25] Gallic acid was used as a standard. The amount of total phenolics was expressed as gallic acid equivalent (GAE) in milligrams per gram dry weight of plant extract.

Measurement of the DPPH radical-scavenging activity: The DPPH free radical scavenging activity was estimated according to the method of Cheung et al. with some modifications.[26] The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. Aliquots of 160 μL of 0.2 mM DPPH in methanol were mixed with 40 μL of each extract (in a concentration of 0.25- 4 mg/mL). The mixture was left under subdued light for 10 min and the absorbance at 520 nm was measured. The results were expressed as IC_{50}, which is defined as the concentration of sample necessary to decrease the absorbance of DPPH in 50%.

$O_2^{\bullet-}$ *Scavenging Activity:* Superoxide dismutase activity was determined according to the method described by Martinez et al. with some modifications.[27] To 1.333 mL of reaction mixture [50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75 μM)] 0.666 mL of extract or ascorbic acid as reference was added. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min of illumination under a fluorescent lamp. The superoxide radical scavenging activity was calculated using the following equation: Scavenging activity (%) = [1 − (As/Ac)] × 100, where As and Ac represents the absorbance of the the extract/standart, control, respectively.

Anti-Lipid Peroxidation (LPO) Activity: Lipid peroxidation was carried out as described by Patro et al. with some modifications.[28] Briefly, small unilamellar vesicles were prepared from phosphatidylcholine (300 mg phosphatidylcholine in 30 mL 10 mM phosphate buffer at pH 7.4 was sonicated on ice for 2 h). To a total volume (1 mL) containing 10 mM potassium phosphate buffer at pH 7.4 (300 μL), the liposome (250 μL), and sample (extract or reference) or control (extraction solvent) (450 μL), was added $FeCl_2$, H_2O_2 and ascorbic acid each in a final concentration of 125 μM. After incubating the mixture at 30 °C for 4 h, 250 μL of the final mixture was added to 500 μL TCA-TBA-HCl reagent (15% w/v, TCA; 0.375% w/v, TBA; 0.25 M HCl), heated in a boiling water bath for 15 min. After centrifugation of the mixture at 3000 *g* for 5 min, the absorbance of supernatant was monitored at 532 nm against blank. Trolox (85 μg) was used as a reference antioxidant. Inhibition of lipid peroxidation (%) by the extract was calculated by using the equation (%) = (1 – S/C) × 100, where C is the absorbance value of the fully oxidised control, S is the absorbance in presence of sample.

Cytotoxic Activity: The cytotoxic activity of the extracts was tested in human cervix adenocarcinoma (HeLa) cell line and normal African green monkey kidney (Vero) cells by using the 3-(4,5-dimethylthiazol-

2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[29] The MTT assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to a colored formazan product by mitochondrial dehydrogenase. The cells were cultured in Eagle's minimum essential medium (EMEM) with Earle's saline, supplemented with an antibiotic-antimycotic mixture [penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL)], and 10% heat-inactivated fetal calf serum at 37 °C in an atmosphere with 5% CO_2. Cells (10^5/mL) were seeded onto the well containing 200 μL EMEM in each well of a 96-well plate and stabilized for 24 h. The stock samples were diluted with EMEM with Earle's saline. The final concentration of DMSO in each sample did not exceed 0.5% v/v. The cells were treated by the extracts (0.001-3 mg) for 48 h. At the end of this period, medium was discarded. To minimize the interference of residues, the adherent cells were washed with phosphate buffer saline then MTT solution (30 μl of 5 mg/mL) was added to each well and incubated 4 hours at 37 ºC. Water-insoluble purple formazan crystals formed in each well were dissolved in 200 μL of DMSO and the resulted optical density (OD) was measured using a microplate reader (μQuant, Bio-Tek) at 570 nm and 690 nm (reference) wavelength. The cell viability was calculated as a percentage of viable cells in extract-treated group vs. untreated control by using the optical density as in following equation:

$$\text{Cell viability (\%)} = \left(\frac{OD_{sample}}{OD_{control}}\right) \times 100.$$

Statistical Analysis: Values are mean ± S.E. of three individual experiments done in triplicates. The data analysis was accomplished using GraphPad Prism, version 5.00 for Windows, GraphPad software Inc., San Diego, CA, www.graphPad.com. The inhibitory concentration (IC_{50}) values were derived from a nonlinear regression model (curve fit) based on sigmoidal dose response curve (variable slope) with coefficient factors between $r^2 = 0.90$ and 0.99.

RESULTS

Total Phenolic Contents: The TPC of the aqueous and methanolic extracts of *Centaurea* sp. is shown in Table I. The highest TPC was observed in the aqueous extract of Cp. In Cs and Cp, aqueous extracts contained approximately two fold phenolics than methanolic extracts, whereas TPC of both extracts were similar in Cc.

DPPH Free Radical Scavenging Activity: The IC_{50} values of the aqueous and methanolic extracts from *Centaurea* sp. are given in Table 1. All extracts showed ignorable DPPH scavenging activity when compared to the reference antioxidant (ascorbic acid). The most effective DPPH scavenger was methanolic extract of Cp, although the other methanolic extracts seemed to be less active than aqueous extracts. No correlation between IC_{50} values and TPC were found. These findings indicate that phenolics do not have a role as a free radical scavenging constituents.

$O_2^{\bullet-}$ *Scavenging Activity:* As shown in Table I, $O_2^{\bullet-}$ scavenging activities of all aqueous extracts were higher than those of methanolic ones. The aqueous extracts of Cc exhibited the highest activity, followed by Cs and Cp with IC_{50} values of 25.91, 68.59 and 73.13 μg/mL, respectively. The methanolic extracts did not exhibit $O_2^{\bullet-}$ scavenging activity, at least for the concentration used in this study except Cp, which was found to be slightly active. These results indicated that the aqueous extracts of Cc had a notable effect on scavenging of superoxide when compared with ascorbic acid, which was used as positive control. This extract seemed to be a potential resource for $O_2^{\bullet-}$ scavenging compounds.

LPO Activity: The lipid peroxidation of liposomes was triggered with $FeCl_2$-ascorbic acid-H_2O_2, and the end-products of the process were measured in terms of the thiobarbituric acid reactive substances (TBARS). The addition of $FeCl_2$-ascorbic acid-H_2O_2 to the liposome for 4 h significantly increased the extent of TBARS formation. *Centaurea* sp. extracts reduced TBARS formation in a dose dependent manner (Figure 1). Methanolic extracts of Cp and Cs showed significant inhibitory effect on TBARS formation. Aqueous extracts of these species were also active but not as strong as methanolic extracts. Interestingly, either aqueous or

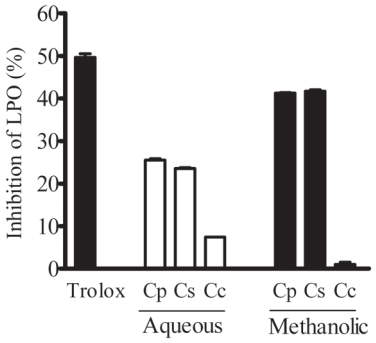

Figure 1. Effect of *Centaurea* sp. extracts (1 mg/mL) and trolox (85 μg/mL) on lipid peroxidation.

methanolic extract of Cc was inactive as an inhibitor of lipid peroxidation.

Cytotoxic Activity: In order to evaluate the cytotoxic activity of the crude methanolic and aqueous extracts of three *Centaurea* species, MTT assays were performed with HeLa and Vero cells. The *Centaurea* sp. extracts decreased the proliferation of HeLa and Vero cells in a dose-dependent manner for 48 h treatment (Figure 2). The most toxic extract on HeLa and Vero cells was the methanolic extract of Cc. The methanolic extract of Cc possess a significant inhibitory effect at of <50 µg/mL concentration and on HeLa and Vero cells with the IC_{50} value of 92.5 and 91.7 µg/mL, respectively. Cytotoxicity of Cc, Cs and Cp extracts on HeLa and Vero cells was investigated for the first time in this study.

DISCUSSION

Many studies have shown that natural antioxidants in medicinal and dietary plants closely related to their biofunctionalities, such as the prevention or suppression of aging and many diseases associated with oxidative stress; cancer, cardiovascular diseases, rheumatoid arthritis, autoimmune diseases and AIDS.[30] Thus, antioxidant capacity is widely used as a parameter to characterize food or medicinal plants. Phenolic compounds have antioxidant properties because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen and hydroxyl radicals.[31] According to the results of obtained from TPC assay, total phenolic content of the aqueous extract of Cp is significantly higher than the other extracts (Table 1). Usually, polar solvents (e.g. methanol, ethanol and water) are used to get the highest extraction yields of phenolic compounds and methanol is the best one, however if glycoside derivatives are present, the aqueous fraction is enriched.[32] All aqueous extracts tested in this study showed high yield of total phenolic content, probably due to the presence of high amount of glycoside derivatives. As a result, the differences in the phenolic content may be attributed to the presence of different extractable components.

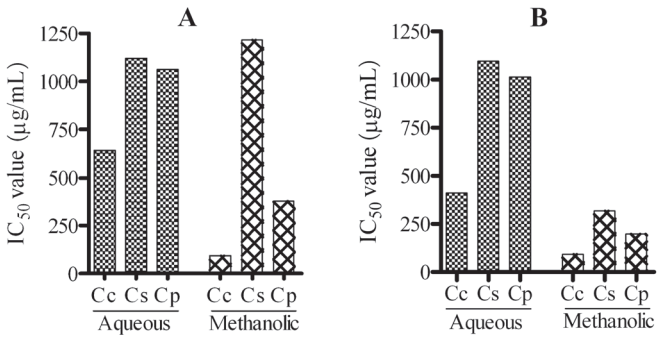

Figure 2. Cytotoxic effect of *Centaurea* sp. extracts on HeLa (A) and Vero (B) cells.

Table 1. Total phenolic contents and antioxidant activities of *Centaurea* sp. extracts

Plants and Standart	Extract Type	TPC (mg GAE/g)	IC_{50} value (µg/mL)	
			DPPH scavenging activity	$O_2^{\bullet-}$ scavenging activity
Cc	Aqueous	37.66 ± 0.63	359.01	25.91
	Methanolic	37.59 ± 0.56	545.61	ND
Cs	Aqueous	32.99 ± 0.07	472.82	68.59
	Methanolic	17.24 ± 1.06	1018.21	ND
Cp	Aqueous	120.50 ± 1.66	479.02	73.13
	Methanolic	72.63 ± 1.84	237.81	187.95
Ascorbic acid	–	–	6.24	7.72

ND: Not determined.

In this study, the antioxidant activity of aqueous and methanolic extracts from *Centaurea* sp. has been evalueted in a series of *in vitro* tests: free radical (DPPH and $O_2^{\bullet-}$) scavenging activity, and lipid peroxidation inhibitory activity. Also, total phenolic contents and cytotoxic activities were investigated.

The results show that all of the three *Centaurea* species have antioxidant potential. DPPH scavenging activity seems to be higher in aqueous extracts of in Cc and Cs, but it is higher in methanolic extract of Cp. The scavenging capacity is correlated to increasing concentration of extracts in all species. It is well known that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g. hydroquinone, pyrogallol, gallic acid) and aromatic amines (e.g. *p*-phenylene diamine, *p*-aminophenol) reduce and decolorize DPPH by their hydrogen donating ability.[33] It appears that *Centaurea* extracts have various constituents which possess hydrogen donating capabilities and act as an antioxidant. The differences between the DPPH radical scavenging activities of different species, seem to be due to the different constituents of the extracts.

Superoxide anion radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species.[30] $O_2^{\bullet-}$ radical plays an important role in formation of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA.[34] Thus, scavenging of this radical is very important in antioxidant defence mechanisms. Table 1 shows $O_2^{\bullet-}$ scavenging activities of *Centaurea* extracts. The aqueous extract of Cc was found to have a strong $O_2^{\bullet-}$ scavenging activity. No correlation between TPC and $O_2^{\bullet-}$ inhibitory activity implies that such activity in all three species is not based on only the presence of polyphenolic antioxidants.[35]

Methanolic extracts of Cp and Cs inhibited the lipid peroxidation. Their inhibitory activities were very close to that of trolox. Aqueous extracts of these species were also active, but not as much as methanolic extracts.

The crude extracts and isolated compounds of some *Centaurea* sp. found to exhibit of antitumor and cytotoxic activity.[18,20-22] Methanolic and aqueous extracts were tested for their cytotoxic activity on HeLa and Vero cell line by the MTT assay. The results indicate that aqueous extracts exhibit very low cytotoxic activity. It was found that methanolic extract of *C. calcitrapa* caused more inhibiton than other methanolic extracts on both cell lines.

In conclusion, this study is the first report on the antioxidant and cytotoxic activities of the crude methanolic and aqueous extracts of selected *Centaurea* species and the results presented here indicate that the selected *Centaurea* species has antioxidant activity, by scavenging free radical, and inhibiting lipid peroxidation. The selected *Centaurea* species can be used as natural antioxidants since especially the aqueous extracts have very low cytotoxic activities. Thus, *Centaurea* species might be a good candidate for further studies targeting the development of new antioxidants. Identification of the antioxidant constituents of these plants and evaluation of their protective effects *in vivo* are in progress. Determination of the antioxidant and cytotoxic effects of *Centaurea* species will promote advanced studies on the identification and quantitation of active constituents that may help to protect consumers against free radical damage and oxidative stress-related diseases.

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