Anais da Academia Brasileira de Ciências (2019) 91(3): e20180462
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201920180462
www.scielo.br/aabc | www.fb.com/aabcjournal

In vitro antioxidant, DNA-damaged protection and antiproliferative activities of ethyl acetate and *n*-butanol extracts of *Centaurea sphaerocephala* L.

AMINA M. LAHNECHE[1,2], RATIBA BOUCHEHAM[2], TEVFIK OZEN[3], MUHAMMED ALTUN[4], NASSIMA BOUBEKRI[1,2], IBRAHIM DEMIRTAS[4], SABRINA BICHA[2], ALI BENTAMENE[2], FADILA BENAYACHE[2], SAMIR BENAYACHE[2] and DJAMILA ZAMA[1,2]

[1]Département de Biologie Animale, Faculté des Sciences de la Nature et de la Vie, Université Frères Mentouri, Constantine 1, Route d'Aïn El Bey, 25000 Constantine, Algérie
[2]Unité de Recherche Valorisation des Ressources Naturelles, Molécules Bioactives et Analyses Physicochimiques et Biologiques, Département de Chimie, Université Frères Mentouri, Constantine 1, Route d'Aïn El Bey, 25000 Constantine, Algérie
[3]Department of Chemistry, Faculty of Science and Arts, Ondokuz Mayis University, Kurupelit Campus, 55139 Samsun, Turkey
[4]Plant Research Laboratory, Chemistry Department, Cankiri Karatekin University, Uluyazı Campus, 18100 Cankiri, Turkey

Manuscript received on May 10, 2018; accepted for publication on August 20, 2018

How to cite: LAHNECHE AM ET AL. 2019. *In vitro* antioxidant, DNA-damaged protection and antiproliferative activities of ethyl acetate and n-butanol extracts of *Centaurea sphaerocephla* L. An Acad Bras Cienc 91: e20180462. DOI. 10.1590/0001-3765201920180462.

Abstract: This study aimed to evaluate the *in vitro* antiproliferative and inhibition of oxidative DNA-damage activities of *n*-butanol (*n*-BuOH) extract of *Centaurea sphaerocephala*. The *in vitro* antioxidant activity of the ethyl acetate (EtOAc) and the *n*-BuOH extracts of this plant were also assayed. To investigate the antioxidant potential, extracts were tested for their capacity to scavenge 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) and to inhibit lipid peroxidation using the TBARs method. The contents of total phenolics and flavonoids were measured. Additionally, antiproliferative activity and DNA-damage inhibition of the *n*-BuOH extract was determined using XCELLigence RTCA instrument and photolyzing 46966 plasmid, respectively. The results exhibited that the scavenging abilities of the EtOAc extract were better than the *n*-BuOH extract with an IC_{50}= 11.59 µg/mL and 16.67 µg/mL for both extracts, respectively. The phenolic and flavonoid contents were found higher in the *n*-BuOH and EtOAc extracts. Furthermore, our results showed that *n*-BuOH extract exhibited a remarkable inhibition of lipid peroxidation with an IC_{50} of 340.94±7.49 µg/mL and had an antiproliferative effect against Hela cells. Extracts of *C. sphaerocephala* showed antioxidant activity on scavenging $DPPH^{\cdot}$. In addition, the *n*-BuOH extract inhibited the lipid peroxidation and exhibited an antiproliferative effect against HeLa cells line (human cervix carcinoma).

Key words: *Centaurea sphaerocephala*, phenolics, antioxidant, lipid peroxidation, antiproliferative, DNA-damaged inhibition.

INTRODUCTION

Plants produce an excessive number of antioxidants to represent a potential source of new molecules and prevent oxidative stress for therapeutic uses (Cragg and Newman 2013). Some medicinal plants have noteworthy potential as natural antioxidants and may be used as protectors against disorders related to oxidative stress (Ting et al. 2011, Bora and

Correspondence to: Tevfik Ozen and Djamila Zama
E-mails: tevfikoz@omu.edu.tr, atrouzl@umc.edu.dz
ORCid: Tevfik Ozen: http://orcid.org/0000-0003-0133-5630
Djamila Zama: http://orcid.org/0000-0002-4312-7701

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Sharma 2011). However, increasing needs for natural antioxidants have provoked great importance for the discovery of powerful free radical scavengers from plants (Khodaie et al. 2012). Some plants showed a radical scavenging capacity dose-dependently and a protective effect on H_2O_2-induced cytotoxicity and DNA damage (Russo et al. 2001). The olive leaf extract, known for its antioxidant capacity, has been confirmed to induce apoptosis in several cancer cells via differentiation steps (Samet et al. 2014). Moreover, *Boerhavia elegans* (Choisy) exhibited strong anti-malarial effect and antioxidant activity due to its high content of phenolic components (Sadeghi et al. 2015). The genus *Centaurea* (trible Cynareae, family Asteraceae) is one of the most widely distributed plant genera in the world. *Centaurea* includes more than 500 species, 45 of which grow spontaneously in Algeria, with 7 species localized in the Sahara region (Ozenda 1958, Quézel and Santa 1963). Several *Centaurea* species are consumed in folk medicine in many countries. For example in Turkey, dried flowers of *Centaurea cyanus* are used as an infusion to relieve diarrhea, gain energy and increase appetite; infusion of *Centaurea calcitrapa* is used as a febrifuge; *Centaurea jacea* is used to increase appetite, to relieve constipation, reduce fever and start menstruation (Reyhan et al. 2004, Baytop 1999). In Tunisia, *Centaurea furfuracea*, an endemic species from the desert region of the North of Africa, is used as astringent and diuretic (Fakhfakh and Damak 2007). While in Algeria, the roots of *Centaurea incana* are used in the region of Aurès for the treatment of liver diseases (Aclinou et al. 1982).

In addition, various studies have shown medicinal properties of *Centaurea* species such as antimicrobial (Karioti et al. 2002), antibacterial (Ćirić et al. 2011), antifungal (Koukoulitsa et al. 2005), cytotoxic (Koukoulitsa et al. 2002, Tukov et al. 2004) and analgesic (Hamid Oudjana 2017). *Centaurea* is well known for its high structural diversity in major bioactive structures, including sesquiterpene lactones, lignans, triterpene and flavonoids (Koukoulitsa et al. 2002, 2005, Tukov et al. 2004, Hamid Oudjana 2017).

Given the interest of *Centaurea* pharmacology and phytochemistry and within the context of the study of *Centaurea* species growing in Algeria Zater et al. 2016, Kolli et al. 2012, Bicha et al. 2011, Bentamene et al. 2010, Medjroubi et al. 2005), we have taken interest in to study *Centaurea sphaerocephala* L. Previous studies on this species and/or its subspecies led to the isolation of flavonoid aglycones (Bentamene et al. 2008), flavonoid glucosides (Bentamene et al. 2010), sesquiterpene lactones (Bruno et al. 1994, Geppert et al. 1983), lignans, sesquilignans and a dithienylacetylene (Bastos et al. 1990). The composition of the essential oil of this species was also reported (Senatore et al. 2006).

Our previous study (Lahneche et al. 2017) showed that *n*-butanol (*n*-BuOH) extract of *C. sphaerocephala* exhibited significant protection against VPA-induced toxicity by its ability to ameliorate the lipid peroxidation and free radical ($DPPH^{\cdot}$) scavenging activity, which enhanced the levels of an antioxidant defense system. This effect could be attributed to its antioxidant properties (Bekhouche et al. 2018).

Therefore, to our knowledge and as a part of our ongoing research program on beneficial health effects of plants and herbs (Boussaha et al. 2015, Lassed et al. 2017), we investigate in the present study, the free radical scavenging activity using $DPPH^{\cdot}$ test, phenol and flavonoid contents in the ethyl acetate (EtOAc) and the *n*-BuOH extracts of *C. sphaerocephala*. *n*-BuOH extract was also assessed for its inhibition of lipid peroxidation, antiproliferative activity, and oxidative DNA damage.

MATERIALS AND METHODS

PLANT MATERIAL AND EXTRACTION

Aerial parts of *C. sphaerocephala* were collected from the area of El Kala, Algeria (21 m, 36° 53' 44"

N, 8° 26' 35" E) in May 2012 and authenticated on the basis of Quézel and Santa (1963) by Professor M. Kaabache, specialist in the identification of Algerian *Centaurea* species (Ferhat Abbas University, Setif 1, Algeria). A voucher specimen (CSA0512-EK-ALG-65) was deposited in the Herbarium of the VARENBIOMOL research unit, Frères Mentouri University, Constantine 1.

The leaves and flowers (2 kg) of this plant were macerated for 24 h, three times with methanol-water (70:30, v/v) at room temperature. After filtration, the clear filtrate was concentrated at 35 °C under vacuum, the remaining solution (400 mL) was dissolved in distilled H_2O (800 mL) under magnetic stirring and kept at 4 °C overnight to precipitate a maximum amount of chlorophylls. After filtrating on a filter paper of Watman no: 1, the resulting solution was extracted with chloroform, EtOAc and *n*-BuOH, respectively. The solutions were dried with sodium sulfate (Na_2SO_4), filtered using normal filter paper and concentrated at 35 °C in a low vacuum to obtain the following extracts: $CHCl_3$ (5.00 g), EtOAc (4.94 g) and *n*-BuOH (34.00 g).

DETERMINATION OF PHENOLIC CONTENTS

The total phenolic contents of the *n*-BuOH and the EtOAc extracts were estimated using Folin-Ciocalteu assay as given by Singleton (Singleton et al. 1999). Folin-Ciocalteu reagent was mixed with the extract or gallic acid (GA) solutions. After 3 min, 3 mL of Na_2CO_3 (20%) was added and then the mixture was incubated at room temperature for 2 hours. The absorbance was measured at 765 nm. All tests were performed in triplicate. GA was used as a standard phenolic compound. The phenolic content was calculated and expressed as a µg GA equivalent per mg of extract (GAE µg/mg extract).

TOTAL FLAVONOIDS

The determination of the total flavonoids in both extracts was estimated by the aluminum chloride colorimetric assay (Wang et al. 2008). A volume of $AlCl_3$ (2%) methanol solution was added to the same volume of sample. After incubation time for one hour at room temperature, the absorbance was then measured at 420 nm. Quercetin was used as a standard compound. The content of total flavonoids was calculated and expressed as a µg quercetin equivalent (QE) per mg of extracts (µg QE/mg extract).

RADICAL SCAVENGING ACTIVITY (DPPH· METHOD)

The activity of the *n*-BuOH and EtOAc extracts was performed using the DPPH method as described by Braca (Braca et al. 2001). This method is based on the principal scavenging of the DPPH to evaluate radical scavenging activity. Briefly, different concentrations of each extract were added to 3 mL of 0.004% of the methanolic solution of DPPH·. The reaction mixture was kept in the dark for a period of 30 min. The absorbance was then monitored at 517 nm. Ascorbic acid was used as a standard. The inhibitory percentage (I, %) of the DPPH· was calculated according to the following equation:

$$I, \% = [(A_{control,\ 517\ nm} - A_{sample,\ 517\ nm})/A_{control,\ 517\ nm}] \times 100$$

where $A_{control}$ is the absorbance of DPPH· solution, A_{sample} is the absorbance of the sample. The IC_{50} of each extract was calculated from the graph plotted of inhibition percentage against the different concentrations of *n*-BuOH and EtOAc extracts. This test was carried out in triplicate at each concentration.

INHIBITION OF LIPID PEROXIDATION

The modified protocol reported by Cao and Ikeda (Cao and Ikeda 2009) was performed to evaluate the capacity of the *n*-BuOH extract to inhibit lipid peroxidation using egg vitellose. As described in our previously published work (Lassed et al.

2015), the assay uses the egg vitellose as a source of lipids. 50 µL of $FeSO_4$ (0.07 M) was mixed with 10% egg vitellose homogenate to induce lipid peroxidation, and then incubated at 37 °C with different concentrations of the extract or ascorbic acid. After a period of incubation for 30 min, 1.5 mL of 2-thiobarbituric acid (TBA, 1%) and 1 mL of trichloroacetic acid (TCA, 20%) were added. The samples were mixed and then incubated at 95 °C for 15 min. After centrifugation, the absorbance of the upper solution was monitored at 532 nm.

The percentage inhibition (I, %) of lipid peroxidation level was calculated using the following equation:

$$I, \% = [(A_{control,\ 532\ nm} - A_{sample,\ 532\ nm})/A_{control,\ 532\ nm}] \times 100$$

where $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of the standard or sample.

THE INHIBITION CAPACITY OF DNA DAMAGE INDUCED BY OXIDATIVE STRESS

The protective capacity of *n*-BuOH extract of *C. sphaerocephala* against DNA damage was tested on 46966 plasmid DNA (extracted from *E. coli*). Plasmid DNA was oxidized with H_2O_2 via UV radiation in the presence or absence of the *n*-BuOH extract and performing agarose gel electrophoresis with the irradiated DNA (Russo et al. 2001). In two polyethylene microcentrifuge tubes, 1 µL aliquots of 46966 plasmid (200 µg/mL) were added, followed by 50 µg of *n*-BuOH extract in one of the two tubes. The other tube was irradiated control (C_R). Then, 4 µL of 3% H_2O_2 was added to each tube. Next, they were placed on the surface of a UV transilluminator (300 nm) during 10 min, directly. In another tube, 1 µL aliquot of 46966 plasmid DNA was placed and served as a non-irradiated control (C_O). All samples were run on 1% agarose gel and then photographed using a Lourmat gel imaging system (Vilber) (Lassed et al. 2015).

EVALUATION OF THE ANTICANCER ACTIVITY OF EXTRACTS USING XCELLigence SYSTEM

Cell Culture and XCELLigence

The Real-Time Cell Analyzer (XCELLigence, RTCA) (ACEABIO, USA) was used for the antiproliferative effect of the *n*-BuOH extract on human cervical cancer (HeLa) cells. The system measured impedance differences to derive cell index values at time points and it may be set by the operator. The cell activity depended on the cell index values and allowed cell behavior in a label-free environment and produced a real-time profile of the cells (Koldaş et al. 2015, Ceyhan et al. 2015). HeLa cells were cultured and supplemented with 10% (v/v) heat-inactivated fetal bovine serum using 2% penicillin-streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. HeLa cells in the culture flask were detached from the bottom of the flask by 10 mL of trypsin-EDTA mixture. The culture medium was added to this cell suspension at the same volume and then mixed gently. The suspension was centrifuged at 600 rpm at Falcon tubes, then removing the upper solution; 5 mL of medium was transferred carefully to the tube and carefully mixed. The concentrations of cell suspensions were determined by CEDEX HIRES Cell Counter using Trypan Blue (Boussaha et al. 2015, Koldas et al. 2015, Ceyhan et al. 2015).

Preparation of E-Plate 96 and Treatment

The culture medium (50 µL) was placed to each well and left in the hood for 15 min. The sterile incubator to allow the electrodes to equilibrate with the culture medium was inserted into the RTCA-SP station. The background measurement was performed for 1 minute to see the condition of the plate (Step 1). The plate was ejected from the station and 100 µL of cell suspension ($2.5x10^4$ cells/100 µL) was added to each well. Three blank wells were left to determine the culture medium.

The plate was also left for another 30 min for the cells to adhere to the bottom and the measurement was performed for 80 min (step 2). At this stage, the cells began to attach to the electrodes on the plate. The cell index value increased as the cells attached to the surface. At the end of the step 2, the different extract solutions (10, 20 and 50 µL equivalent to 50, 100 and 250 µg/mL concentrations, respectively) were added to the wells and the final volumes were completed to 200 µL. Lastly, the plate was placed into the station and a measurement lasting 48 h was started (Step 3). During step 3, the status of the cells was recorded every 10 min with XCELLigence RTCA instrument. This measurement was made in triplicate (Boussaha et al. 2015, Koldas et al. 2015, Ceyhan et al. 2015). In apparel, cis-platin as a standard control at the doses of 250, 100, 50 and 10 µg/mL was used by following the same steps.

STATISTICAL ANALYSIS

Data are expressed as a mean ± standard deviation (SD). The statistical interferences were based on student's test for mean values and compare to standard. Differences were highly significant at $p < 0.01$ and significant at $p < 0.05$.

RESULTS AND DISCUSSION

TOTAL PHENOLIC AND FLAVONOID CONTENTS OF THE EtOAc AND THE *n*-BuOH EXTRACTS

The total phenolic content of *C. sphaerocephala* was higher in the EtOAc extract: 357±11.32 µg GAE/mg extract compared to the *n*-BuOH extract: 202.5±1.5 µg GAE/mg extract. However, the amounts of flavonoids in the EtOAc and the *n*-BuOH extracts were 283.2±4.17 and 273.8±5.38 µg QE/mg extract, respectively. The high amount of total phenol content in both extracts justified their high antioxidant activities. Therefore, the antioxidant activity of plant extracts may result from phenolic compounds. Similar results were found in other studies using different plants: *Caesalpinia bonducella* seeds (Shukla et al. 2009), *Chromolaema odorata* leaves (Rao et al. 2010), biorefining of *Bergenia crassifolia* root and leaves (Kraujalienė et al. 2016); different populations of lavandin (Bajalan et al. 2016) and some plants from semiarid Mexican region (Wong-Paz et al. 2015). A lignin, arctigenin, and a number of flavonoids, 3-*O*-glucosyl-isorhamnetin, rutin, 6-*C*-glucosyl-luteolin, isoquercitrin, trifolin, isoscoparin and isovitexin were previously isolated from aerial parts of *C. macrocephala* (Sarker et al. 1997, Ribeiro et al. 2002). The isolation, identification, toxicity and antioxidant activities of the isolated lignans such as arctiin, matairesinoside, matairesinol and lappaol A from seeds of *C. macrocephala* were also reported (Shoeb et al. 2004).

FREE RADICAL SCAVENGING ACTIVITY OF THE EtOAc AND THE *n*-BuOH EXTRACTS

$DPPH^{\cdot}$ has been used to determine free radical scavenging capacity of plant extracts (Erenler et al. 2014). As showed in Fig. 1, the scavenging ability of the EtOAc and the *n*-BuOH extracts was concentration-dependent (Erenler et al. 2014). The IC_{50} values were calculated as the concentration of the sample that caused the inhibition of 50% of free radical ($DPPH^{\cdot}$). A lower IC_{50} value indicated a higher scavenging activity. In this study, both extracts proved to be effective scavengers of $DPPH^{\cdot}$. The IC_{50} values of ascorbic acid, EtOAc and *n*-BuOH extracts were 5.18±0.12 µg/mL, 11.59±0.04 µg/mL and 16.67±0.11 µg/mL, respectively.

LIPID PEROXIDATION INHIBITORY ACTIVITY OF THE *n*-BuOH EXTRACT

In the current work, we investigated the potential of the *n*-BuOH extract of *C. sphaerocephala* in inhibiting the lipid peroxidation induced by the $FeSO_4$ system, using egg vitellose homogenate. As shown in Fig. 2, the IC_{50} values of the

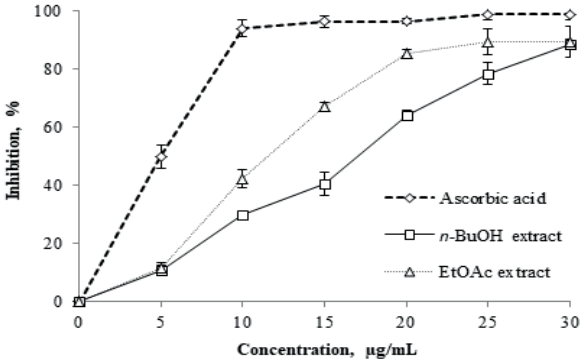

Figure 1 - Free radical scavenging capacity of EtOAc and *n*-BuOH extracts of *C. sphaerocephala* using DPPH.

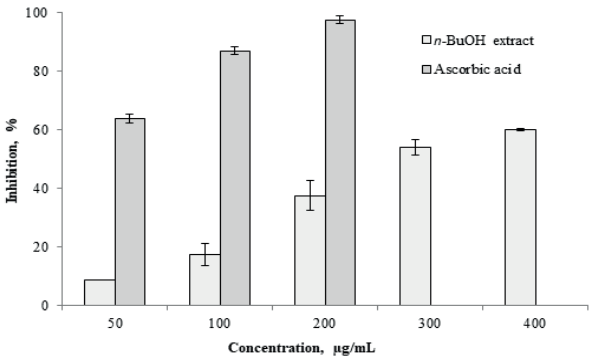

Figure 2 - Inhibition of lipid peroxidation induced by *n*-BuOH extract of *C. sphaerocephala* and ascorbic acid.

inhibitory effect of the extract and ascorbic acid were 340.94±7.49 µg/mL and 20.62±0.93 µg/mL, respectively. The percentage inhibition of lipid peroxidation of the *n*-BuOH extract of *C. sphaerocephala* (100 and 200 µg/mL) was found to be 17.36% and 37.50%. While the ratio at the same concentrations for ascorbic acid was found to be 86.95% and 97.50% ,respectively. The *n*-BuOH extract decreased lipid peroxidation through its antioxidant and anti-radical power. In biological systems, lipid peroxidation generates a number of cytotoxic products, such as MDA, a marker of the oxidative stress in tissues injury (Zama et al. 2007).

DNA DAMAGE INHIBITION EFFICIENCY OF THE *n*-BuOH EXTRACT

Several studies provide evidence that DNA is susceptible to oxidative damage induced by free radicals in several diseases such as cancer. In order to investigate the protective effect of the *n*-BuOH extract of *C. sphaerocephala*, the UV-photolysis of H_2O_2 test was performed (Fig. 3). The untreated nonirradiated DNA (C_O) generated one band corresponding to supercoiled circular DNA which the native form is normally found *in vivo*. While both the irradiated control and the test sample (containing 50 µg of the *n*-BuOH extract) showed two new forms of DNA: the relaxed circular and the linear forms. These forms naturally occur when the DNA is nicked and damaged. In our case, the

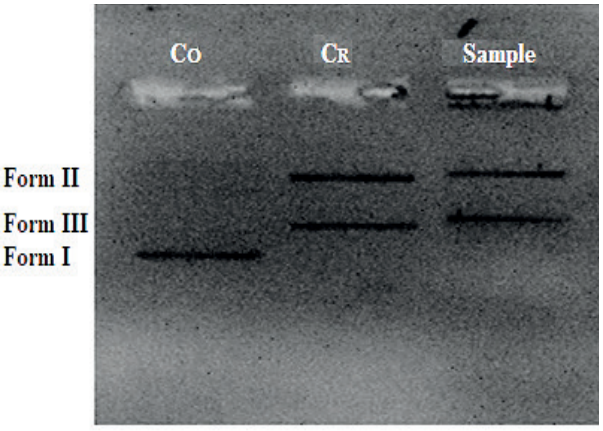

Figure 3 - Effect of *n*-BuOH extract of *C. sphaerocephala* on the safety of 46966 plasmid DNA against oxidative damage caused by UV-photolysed H_2O_2.**C_o:** untreated non-irradiated DNA.**C_R:** untreated UV-irradiated DNA Sample**:** DNA and UV-irradiated treated with *n*-BuOH extract. **Form I:** supercoiled plasmid DNA.**Form II:** open circular double stranded DNA. **Form III:** linear DNA

extract did not show any ability in protecting DNA against UV photolysis of H_2O_2 induced damage and did not keep DNA in its native supercoiled form (Ozaki et al. 2015, Valko et al. 2001).

THE ANTIPROLIFERATIVE ACTIVITY OF THE *n*-BuOH EXTRACT

HeLa (human cervix carcinoma) cells were the first human cell line established in culture (Lucey et al. 2009) and meanwhile has become the most widely used human cell line in biological research (Manosroi et al. 2012). The antiproliferative

activity of the *n*-BuOH extract against HeLa cells was tested using XCELLigence RTCA software (Koldaş et al. 2015). In this study, antiproliferative effects of the *n*-BuOH extract obtained from *C. sphaerocephala* L. were examined on HeLa cell lines. The cis-platin as metal-based drug candidate molecules was proved to have antiproliferative activity. The last fifty years, many metal complexes such as cis-platin were carried out *in vitro* and *in vivo* biological assays. Therefore, the cis-platin was used as a positive control in this study. As represented in Figs. 4 and 5, in the first 80 minutes, cells not treated with extract or positive control (cis-platin) were observed to have normal growth, but the medium wells (green line) did not have any increase. After step 2, the extract or the positive control was added to the cell culture media of each well of E-plate 96 at different concentrations.

Antiproliferative activity of the *n*-BuOH extract at 100 and 50 µg/mL concentrations showed lower activity than 250 µg/mL. As shown in Fig. 4, the lowest activity (turquoise line) was observed at the lowest concentration of 50 µg/mL. The low concentration decreased antiproliferative activity stepwise after the first 10 h and cell index (CI) increased slowly during the period. However, CI values of low concentration were lower than control value (red line). The antiproliferative effect of 100 µg/mL concentration (pink line) was not as high as the highest dose but more efficient than a lower dose. The extract showed an interesting effect against HeLa cells especially between the 30 to 50 h. The best effects observed from *n*-BuOH extract at the higher concentration of 250 µg/mL compared to the positive and the negative controls and literature (Oke-Altuntas et al. 2016, Yaglıoglu et al. 2013).

These results showed that the extract exhibited different effect against HeLa cell line at different concentrations. Thus, the antiproliferative activity of the *n*-BuOH extract increased in a dose-depended manner from 50 to 250 µg/mL. It may be suggested that the antiproliferative properties of the *n*-BuOH extract of *C. sphaerocephala* could be attributed to the presence of phenolic compounds, especially flavonoids reported in previous study (Bruno et al. 1994). This class of natural compounds plays an important role in cancer prevention and treatment (Samet et al. 2014, Koldaş et al. 2015, Abay et al. 2015). Many studies reported that flavonoids have protective effects against some diseases as cancer. Flavonoids as isocoreopsin, butrin and isobutrin

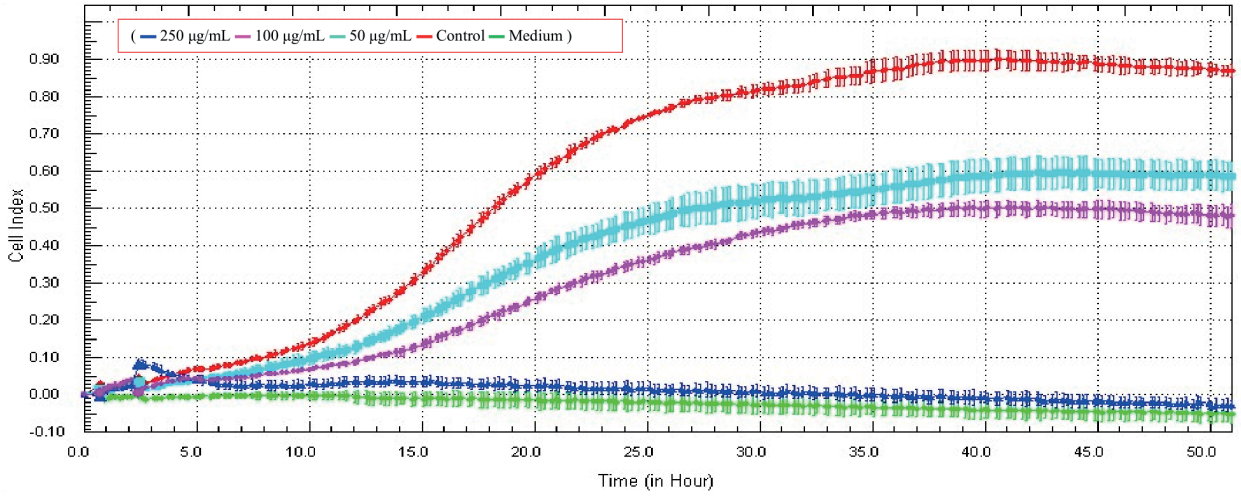

Figure 4 - *In vitro* antiproliferative activity of *n*-BuOH extract of *C. sphaerocephala* against HeLa cell lines ($2.5x10^4$ cell/well). Each substance was tested twice in triplicates using RTCA xCELLigence instrument. Different concentrations of *n*-BuOH extract were applied to the cells represented by different color (250 µg/mL 100 µg/mL 50 µg/mL Control Medium).

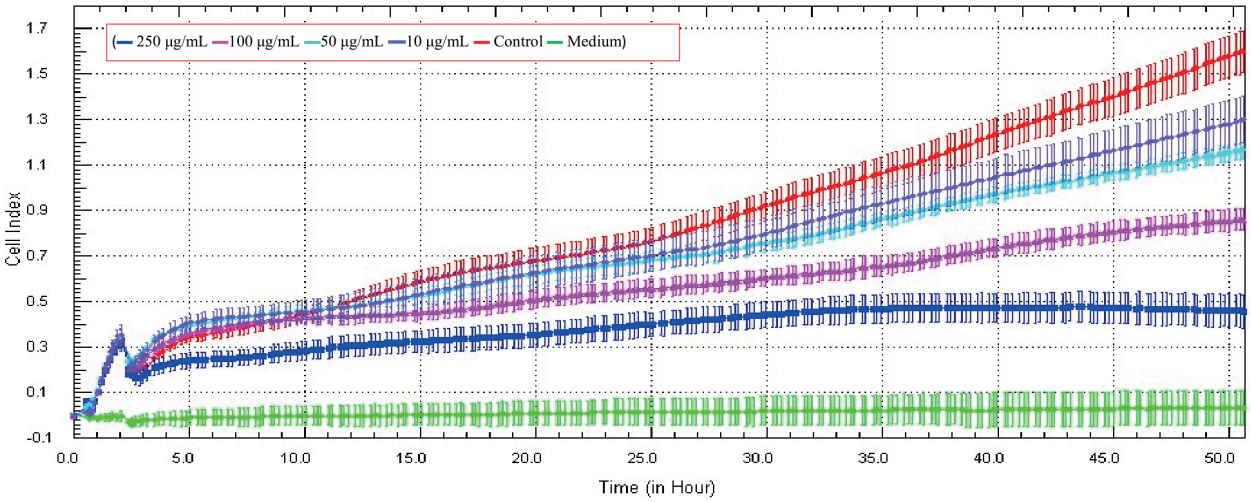

Figure 5 - *In vitro* antiproliferative activities of cis-platin at different concentrations indicated with colors (250 µg/mL 100 µg/mL 50 µg/mL 10 µg/mL Control Medium).

have an essential role in the chemoprevention of colon cancer (Teoh et al. 2017). Plant polyphenols have drawn increasing attention due to their protective effects against several oxidative stress associated diseases such as cancer. Natural phenolics have been found to mediate all stages of cancer development (Subramaniyan et al. 2016, Yildiz et al. 2017). In addition to their antioxidant activities, they inhibit cancer development via a number of cellular essential mechanisms (Dai and Mumper 2010).

These results confirmed other studies which reported that *Centaurea* species contain natural potent anticancer agents against a variety of human malignances including prostate, lung, colon, stomach, kidney, pancreas and mammary glands (Yaglioglu et al. 2014). This beneficial effect has been attributed to the presence of high amounts of polyphenols, which are potent antioxidants especially sesquiterpene lactones (Erenler et al. 2016, Demirtas et al. 2013).

CONCLUSION

In the present study, both extracts of *C. sphaerocephala* showed strong antioxidant activity on scavenging of DPPH. Their high antioxidant property might be due to the presence of polyphenols and flavonoids. The *n*-BuOH extract inhibited lipid peroxidation. In addition, the *n*-BuOH extract showed antiproliferative activity against HeLa cell lines. It can be concluded that the *n*-BuOH extract of *C. sphaerocephala* L. has antiproliferative components. The strong antiproliferative activity at the high concentration of this extract suggests that this plant may be a promising source of natural antioxidant and functional plant material. These findings need extensive studies on the isolation and the determination of chemical profiles and the mechanism of antiproliferative and antioxidant activities.

ACKNOWLEDGMENTS

The authors thank the Algerian Ministry of Higher Education and Scientific Research (MESRS) for financial support. The authors also thank Professor M. Kaabeche for the identification of the plant material and Professor S. Khennouf for improving the English quality of this manuscript.

AUTHOR CONTRIBUTIONS

Amina Maya Lahneche performed the experiments, acquisition of data, drafting of manuscript. Ratiba

Boucheham contributed in plant extraction procedure. Tevfik Ozen contributed in the analysis of the data, in critical reading and draft of the manuscript. Muhammed Altun contributed to the antiproliferative activity of the extract. Nassima Boubekri contributed in the antioxidant assay. Ibrahim Demirtas contributed to experimental design, conducting studies and to critical reading and draft of the manuscript. Sabrina Bicha contributed in plant extraction procedure. Ali Bentamene contributed in plant extraction procedure. Fadila Benayache contributed in conceived and designed the experiments, analysis and interpretation of data, drafting of manuscript, critical revision. Samir Benayache contributed in chose and collected the plant material, analysis and interpretation of data, drafting of manuscript, critical revision. Djamila Zama contributed in study conception and design, analysis and interpretation of data, drafting of manuscript, critical revision, all authors read, approved and revised the final manuscript.

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