



BJP

Bangladesh Journal of Pharmacology

Research Article

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Anti-oxidant, DNA-damage protection and anti-cancer properties of *n*-butanol extract of the endemic *Perralderia coronopifolia*

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Article Info

Received: 10 October 2017

Accepted: 19 February 2018

Available Online: 15 March 2018

DOI: 10.3329/bjp.v13i1.34255

Cite this article:

Bekhouche K, Ozen T, Boussaha S, Koldas S, Yenigun S, Lassed S, Demirtas I, Benayache F, Benayache S, Zama D. Anti-oxidant, DNA-damage protection and anti-cancer properties of *n*-butanol extract of the endemic *Perralderia coronopifolia*. Bangladesh J Pharmacol. 2018; 13: 82-89.

Abstract

This study was designed to evaluate *in vitro* the total phenolic, flavonoid content, anti-oxidant activity, oxidative DNA-damage protection and anti-cancer activity of *n*-butanol extract from *Perralderia coronopifolia*, endemic plant. DNA protection capacity was performed using 46966 plasmid DNA against UV-photolysis of H₂O₂-induced oxidative damage. The anti-proliferative effects of extract were performed on HeLa and C6 cells. Experimental results showed that extract had convenient number of phenolic and flavonoids. Furthermore, it provided strong anti-oxidant, reducing power, hydrogen peroxide and higher DPPH· scavenging ability with IC₅₀= 7.02 ± 0.02 µg/mL. As it shown an oxidative plasmid DNA-damage inhibition against UV-photolysis of H₂O₂, an anti-cancer activity at higher concentrations in cells. The data obtained that *P. coronopifolia* extract could be useful in human protection against infection and degenerative illness.

Introduction

Oxidative stress is the most important factor in the stimulation of certain chronic and degenerative illnesses. DNA damage is the important manifestation of long term ROS exposure, thereby inducing a various disorder in humans (Souri et al., 2008). Some findings suggest that DNA damage may be a major factor risk for several pathogenesis and deceases such as neurodegenerative (Lezza et al., 1999) and cardiovascular diseases (Collins et al., 1998), as well as diabetes (Dandona et al., 1996) and cancer (Kumar et al., 2012).

Anti-oxidants from natural or synthetic products provide novel possibilities to protect and treat oxidative damage by inhibiting or scavenging free radicals (Casa-

grande et al., 2006). The investigation of several parts of phenolic plants like fruit, vegetable, nut, seed, leaf, root and bark established their potent pharmacological activities and medicinal properties.

The current study report on *P. coronopifolia* (Asteraceae). It is one of the Algerian endemic species which is seemed to be used as a natural therapeutic remedy, grow in the elevated regions in the North-Western of Africa (Algeria, Morocco and Libya) (Boussaha et al., 2015). Considering the traditional therapeutic use of this plant, it is necessary to evaluate its biological and oxidative potentials.

Therefore, the main purpose of this study was to investigate the anti-oxidant and the anti-cancer pro-



properties of *n*-butanol extract from *P. coronopifolia* using *in vitro* models' systems to know its consumption of total phenolic content and flavonoids. As well as its anti-oxidant activity, reducing power, hydrogen peroxide scavenging, DPPH· scavenging and metal chelating activities. Furthermore, our study reports the inhibition of oxidative 46966 plasmid DNA-damage against UV-photolysis of H₂O₂, and the inhibition of proliferation of different cancer-cell lines.

Materials and Methods

Chemicals

The chemicals utilized in the tests were in analytical grade and purchased from the Sigma-Aldrich and Roche.

Plant material

The plant material (*P. coronopifolia*) was collected in 2011 at an elevated region located around Taghit in the South-West of Algeria. It was authenticated by M Mouhamed Ben Abdelhakem, manager of the wildlife preserving organization of Bechar. A label sample (PCA0511-TAG-ALG-52) was put at the herbarium of VARENBIOMOL Unit, University of Mentouri1, Constantine.

A dried aerial parts (1,400 g) of the plant were macerated for 48 hours at room temperature with ethanol-water (80:20, v/v), three often. The rest was concentrated and liquefied in 650 mL of water, after filtration. The resultant mixture was treated by chloroform, ethyl acetate and *n*-butanol, respectively. The organic phases were dried over Na₂SO₄ and filtered by common filter paper and concentrated in vacuum at ambient temperature to get the chloroform (2 g), ethyl acetate (7 g) and *n*-butanol (40 g) extracts.

Anti-oxidant properties

Determination of total phenolic and flavonoid contents

Total flavonoid was measured by the Folin-Ciocalteus reagent (FCR) according to the method described by Singleton et al. (1999) using gallic acid (GA) as a standard phenolic compound. 100 µL of FCR and 1.6 mL of distilled water were mixed with 20 µL of extract. After 3-8 min, 0.3 mL of Na₂CO₃ was added. The absorbance of sample was read at 765 nm after 30 min of incubation at 37°C. The total phenolic compounds concentration of extract was quantitated as µg of gallic acid equivalent (GAE)/mg plant material with standard deviation.

Total flavonoid content was determined as described in a reference (Wang et al., 2008) using quercetin as standard. 0.5 mL of sample was added to of AlCl₃ (2%; 0.5 mL). The absorbance of sample was read at 420 nm lasting 1 hour of incubation. The quercetin was used as

a standard. The content of flavonoid compounds in extract was estimated as µg quercetin equivalent (QE)/mg plant material with standard deviation.

Determination of total anti-oxidant activity

The activity of the *n*-butanol extract was determined spectrophotometrically as mention in the phosphomolybdenum method (Prieto et al., 1999). This assay founded on the potent of reducing Mo(VI) to Mo(V) by the extract analyzed and the resulting of green phosphate/Mo(V) substances. The extract solution (0.3 mL) was mixed with 2.7 mL of the reagent solution prepared in ammonium molybdate (4 mM), sulfuric acid (0.6 M) and sodium phosphate (28 mM). The sample and standard solutions were left in a water bath at 95°C during 90 min. After cooling the samples to room temperature, the absorbance of the resulting green sample-phosphomolybdenum complex was read at 695 nm. For the blank, just a replacement of sample by 0.3 mL of ethanol. A typical blank solution contained 2.7 mL of reagent solution, after it was left under the similar conditions as sample. The activity was defined as the absorbance of the sample and standard. A higher absorbance value is indicative a higher anti-oxidant capacity.

Reducing power assay

The reducing power of *n*-butanol extract and standard anti-oxidants was measured spectrophotometrically using the method of Oyaizu (1986). This assay established on the reduction of the Fe⁺³-ferricyanide to the Fe⁺², by the reductant compounds in the mixture. 1 mL of the sample solutions (50-500 µg/mL) were added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1%; 2.5 mL). The mixtures were placed in a water bath at 50°C pending 20 min. The mixtures were centrifuged at 1000 x g for 20 min after adding of trichloroacetic acid (10%; 2.5 mL). Finally, the supernatant (2.5 mL) was added to 2.5 mL of distilled water and ferric chloride (0.5 mL; 0.1%). The mixture intensity of the bleu-green color was read at 700 nm. In this assay, the upper absorbance of sample indicates better reducing power under the reaction conditions.

H₂O₂ scavenging activity

H₂O₂ scavenging activity was measured as a slightly modification described by Zhao et al. (2006). 1 mL of sample was mixed with H₂O₂ (0.1 mM; 1 mL), ammonium molybdate (3%; 100 µL), H₂SO₄ (2 M; 10 mL) and KI (1.8 M; 7 mL). Finally, the mixture was titrated using Na₂S₂O₃ (5 mM) till disappearing yellow color. The reaction without sample was used like a control. The percentage of scavenging effect was calculated using the formula given below:

$$\text{H}_2\text{O}_2 \text{ scavenging activity, \%} = [(V_{\text{control}} - V_{\text{sample}}) / V_{\text{control}}] \times 100$$

Where, V_{control} is the volume of Na₂S₂O₃ and V_{sample} is the

volume of Na₂S₂O₃ solution H₂O₂ added to the test samples

DPPH· scavenging assay

DPPH· scavenging assay was performed according to the method in the literature (Braca et al., 2001). 3 mL of a methanol solution of DPPH· were mixed with 1 mL of sample in various concentrations (1-20 µg/mL). After 30 min incubation in the dark at room, absorbance was read at 517 nm. The inhibition percentage was determined using the formula:

$$\text{Inhibition, \%} = (1 - A_1/A_0) \times 100$$

Where, A₁ is absorbance of sample and A₀ is absorbance of control

Metal chelating activity

The Fe²⁺ chelating activity of extract and standard antioxidants was measured as a mentioned assay by Dinis et al. (1994). The different concentrations (50-500 µg/mL) of the sample were added separately to 2 mM of FeCl₂ (0.05 mL) and left for incubation at room temperature for 5 min. The reaction was started by adding the ferrozine (5 mM; 0.3 mL). After shaking forcefully, the mixture was incubated at room temperature pending 10 min. EDTA was used as a positive control. Absorbance was read at 562 nm. The inhibition percentage of Fe²⁺-ferrozine complex creation was measured as the next formula:

$$\text{Chelating capacity, \%} = [(A_0 - A_1)/A_0] \times 100$$

Where, A₀ is the absorbance of the Fe²⁺-ferrozine complex and A₁ is the absorbance of the test solutions

DNA damage inhibition efficiency

DNA protection capacity of the test plant extract was performed as described by Russo et al. (2001), using photolyzing 46966 plasmid DNA (extracted from *E. coli*) through UV radiation in the presence of H₂O₂ and the performance of agarose gel electrophoresis with the irradiated DNA. 1 µL aliquot of 46966 plasmid (20 µg/mL) was put in polyethylene microcentrifuge tube, then 50 µg of *n*-butanol extract plant was added to single tube. One tube did not contain the extract was served as an irradiated control (C_R). Then, 4 µL of H₂O₂ (3%) was added into the tube (including C_R). Lastly, tubes were put on the outer face of a UV transilluminator (300 nm) and irradiated at room temperature during 10 min. Individual tube contains 1 µL aliquots of 46966 plasmid DNA was placed also, which performed as the non-irradiated control (CO). Samples were run on agarose gel (1%) and photographed using a Lourmat gel imaging system (Vilber).

Anti-cancer activity

The xCELLigence RTCA-SP (Real Time Cell Analyzer-Single Plate) instrument is a grouping of four parts: An E-Plate 96, a plate station, an analyzer and a computer

including RTCA software. It was used to evaluate the antiproliferative effects of the *n*-butanol extract of *P. coronopifolia* on HeLa and C6-cell lines (Demirtas et al., 2009).

HeLa (human cervix carcinoma) and C6 (rat brain tumor) cells were developed in DMEM-HG (Dulbecco's modified eagle's medium) with 10% (v/v) FBS (fetal bovine serum) and 2% penicillin/streptomycin solution, at 37°C in a humidified atmosphere of 5% CO₂.

HeLa and C6-cells were detached from bottom of the culture flask using 10 mL trypsin-EDTA mixture. After detaching, the same volume of culture medium was placed in the flask and mixed thoroughly and put in centrifuge 600 rpm for 5 min (Nüve NF 800, Turkey). 5 mL of medium was mixed carefully with the supernatant. The concentration of cells in this suspension was determined using CEDEX HIRES Cell Counter.

50 µL of the medium was placed to every well of E-Plate 96 and left in the sterile cabinet for 15 min, then incubated for 15 min. 100 µL of the cell suspension (2.5 × 10⁴ cells/100 µL) was placed into wells. Exceptionally, 100 µL of 5-fluorouracil (5-FU) was added as a control in the last wells. The plate incubated in the hood for 30 min in order to adhere cells to the bottom, then attached to xCELLigence RTCA-SP station in the incubator. A measurement during 80 min was started. The extract solutions (50, 100 and 250 µg/mL) in DMSO were injected to the wells and adjusted until 200 µL using culture medium. The control and medium wells did not have any extract solutions. Lastly, the plate was placed in the incubator xCELLigence instrument and measurements were taken over 48 hours.

Statistical analysis

Data were presented as mean ± standard deviation of the mean of three parallel measurements. The analyses of results were performed by using the SPSS 18 Software and evaluated by t-test.

Results

The content of total phenolics and flavonoids

The *n*-butanol extract of *P. coronopifolia* contained high phenolic compounds (444.0 ± 0.9 µg GAE/mg plant material) and flavonoids (400.3 ± 0.5 µg quercetine equivalents/mg plant material).

Total anti-oxidant activity

The extract has a high anti-oxidant activity, but it was inferior to those of anti-oxidant standards (Table I). All samples exhibited a concentration-dependent total antioxidant activity. At 500 µg/mL, *n*-butanol extract exerted a strongest anti-oxidant activity (3.0) and followed

Table I				
<i>In vitro</i> anti-oxidant activity of <i>P. coronopifolia</i> extract				
Sample	50 µg/mL	100 µg/mL	250 µg/mL	500 µg/mL
Total anti-oxidant activity, 695 nm				
<i>n</i> -Butanol extract	0.03 ± 0.01	0.2 ± 0.01	1.5 ± 0.06	3.0 ± 0.1
Ascorbic acid	0.04 ± 0.02	0.3 ± 0.01	1.0 ± 0.07	2.7 ± 0.02
Butylated hydroxyanisole	0.07 ± 0.01	0.2 ± 0.02	0.7 ± 0.02	1.5 ± 0.06
Reducing power, 700 nm				
<i>n</i> -Butanol extract	0.2 ± 0.01	0.1 ± 0.01	0.2 ± 0.01	0.4 ± 0.01
Ascorbic acid	0.5 ± 0.005	1.1 ± 0.03	3.5 ± 0.2	3.8 ± 0.06
Butylated hydroxyanisole	0.3 ± 0.01	0.6 ± 0.02	1.2 ± 0.1	3.5 ± 0.05
Trolox	0.2 ± 0.01	0.4 ± 0.01	0.9 ± 0.05	1.9 ± 0.1
Hydrogen peroxide scavenging activity, %				
<i>n</i> -Butanol extract	31.7 ± 3.2	38.9 ± 2.7	47.6 ± 0.87	65.6 ± 0.5
Ascorbic acid	17.5 ± 0.8	34.1 ± 2.1	54.0 ± 0.9	66.7 ± 0.6
α -Tocopherol	30.2 ± 0.9	38.1 ± 0.9	47.6 ± 3.6	55.6 ± 0.8
Butylated hydroxyanisole	25.4 ± 3.2	41.3 ± 3.9	50.8 ± 4.1	60.3 ± 3.8
Trolox	31.7 ± 2.1	41.3 ± 0.8	46.0 ± 2.7	50.8 ± 0.6
Metal chelating activity, %				
<i>n</i> -Butanol extract	7.9 ± 1.3	29.2 ± 0.7	41.7 ± 2.0	50.9 ± 1.2
Ethylenediaminetetraacetic acid	10.2 ± 1.3	21.9 ± 0.7	37.5 ± 3.0	45.9 ± 1.2
Ascorbic acid	6.5 ± 2.7	10.6 ± 0.5	23.2 ± 1.2	40.6 ± 0.8
α -Tocopherol	6.2 ± 1.3	8.9 ± 1.8	20.2 ± 2.1	23.6 ± 3.2
Butylated hydroxyanisole	2.1 ± 0.4	21.1 ± 0.7	36.8 ± 3.7	52.6 ± 1.8

by ascorbic acid (2.7), and BHA (1.5).

Reducing power activity

The *n*-butanol extract showed a stronger reducing power comparable to those of anti-oxidant standards used as positive controls: Trolox, BHA, α -tocopherol and ascorbic acid (Table I). It increased with increasing concentrations, ($p < 0.05$) significantly.

H₂O₂ scavenging activity

The H₂O₂ scavenging activity of *n*-butanol extract was compared with ascorbic acid, α -tocopherol, BHA and trolox (Table I). It was determined that the hydrogen peroxide scavenging activities of the samples increased depending on the different concentrations. The H₂O₂ scavenging activity of *n*-butanol extract was higher than that of α -tocopherol, BHA and trolox ($p < 0.05$).

DPPH[•] scavenging activity

The measurements of DPPH[•] scavenging capacities of *n*-butanol extract and ascorbic acid as standard radical scavenger indicated in the order of ascorbic acid > *n*-butanol with IC₅₀ value of 5.0 ± 0.1 and 7.0 ± 0.02 µg/mL, respectively (Figure 1). The activity of extract was close to that of ascorbic acid.

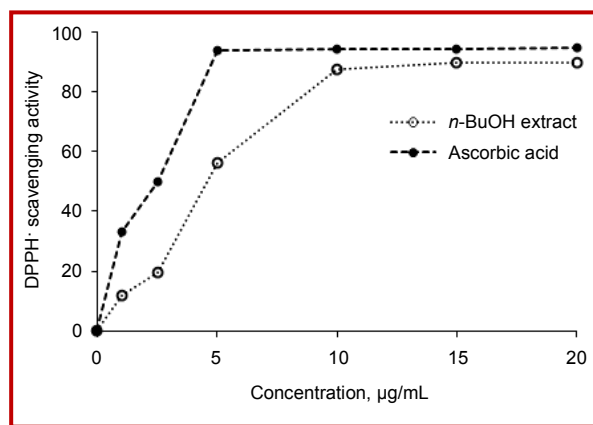


Figure 1: The free radical (DPPH[•]) scavenging of the *P. coronopifolia n*-butanol extract

Metal chelating activity

The *n*-butanol extract of *P. coronopifolia* was able to chelate Fe²⁺ in a concentration-dependent manner (Table I). At 100 and 250 µg/mL, the metal chelating activity of extract showed higher than that of BHA and ascorbic acid. Except for BHA, the extract exhibited higher chelating activity than standards at 500 µg/mL.

DNA damage inhibition efficiency

Results showed that the extract at a dose of 50 μg was exhibited a completed protection of plasmid DNA. The circular (C) form of untreated DNA was converted into nicked form: Relaxed (R) and/or linear (L), which indicates the plasmid-DNA damaging. While the upon-treatment with *n*-butanol extract of *P. coronopifolia* regained the DNA to its native circular form (Figure 2).

Anti-cancer activity

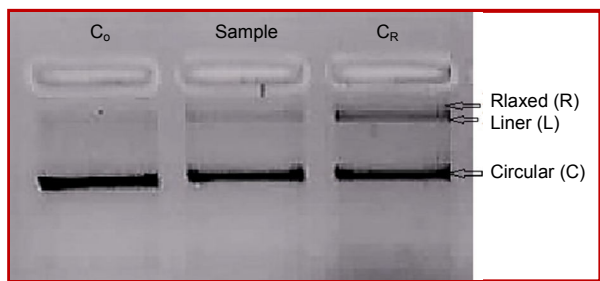


Figure 2: Effect of anti-cancer activity (50 μg) against oxidative to 46966 plasmid DNA damage caused by UV-photolysis of H_2O_2 (3%, v/v) C₀ = untreated non-irradiated DNA; Sample = DNA UV-irradiated treated with *n*-butanol extract of *P. coronopifolia*; C_R = untreated UV-irradiated DNA

Figure 3 exhibits the diagram of the anti-cancer test with deviation bars. In the present work as compared with the positive control, the extract revealed diverse profiles at diverse concentrations (250, 100 and 50 $\mu\text{g}/\text{mL}$). As seen in diagram Figure 3A, during the first two hours cells not treated with extracts were observed a normal growth. 24 hours post-treatment, HeLa cells were inactivated by *n*-butanol extract and showed the maximum activity through the concentration of 250 $\mu\text{g}/\text{mL}$. Lower activities were obtained at lower concentrations (100 and 50 $\mu\text{g}/\text{mL}$), nearly same with control during the same period of time.

However, profiles exhibited dissimilarities at various time points, which lower concentrations indicated certain positive inhibition-activities against C6 cells-proliferation during 27 hours post-treatment. As shown in Figure 3C, after a short time they are missing their capacities of inhibition.

Discussion

The anti-oxidant activity of extract is the result of an existence of polyphenols and flavonoids, which possibly act upon by offering electrons and free radicals. Similar finding was obtained by Barros et al. (2007) and Ozen et al. (2011) on their studies on different extracts. Flavonoid compounds are the most essential plant elements by the reason of their $\cdot\text{OH}$ groups which can offer the anti-oxidant capacity (Kumar et al., 2008).

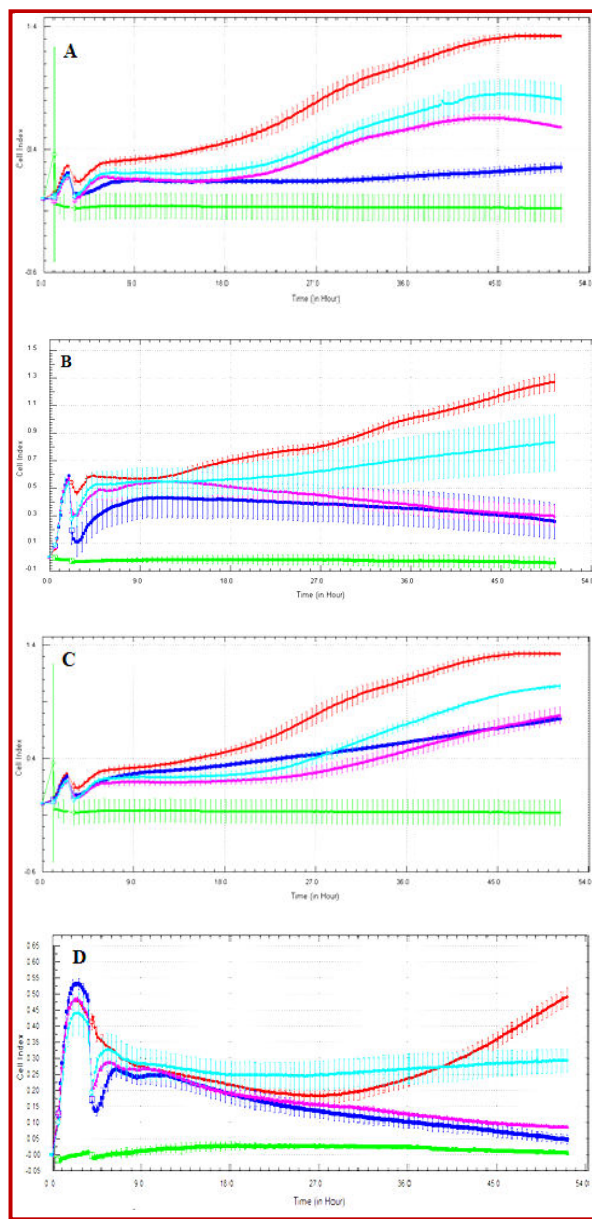


Figure 3: The anti-cancer activity of the *n*-butanol extract of *P. coronopifolia* against HeLa and C6 (2.5×10^4 cell/well) cells line at several extract concentrations (— 50 $\mu\text{g}/\text{mL}$, — 100 $\mu\text{g}/\text{mL}$, — 250 $\mu\text{g}/\text{mL}$, — Control, — Medium). Proliferation assay of the *n*-butanol extract of *P. coronopifolia* (A) and the 5-fluorouracil as a positive control (B) against HeLa cells; Proliferation assay of the *n*-butanol extract of *P. Coronopifolia* (C) and the 5-fluorouracil as a positive control (D) against C6 cells

They have been shown to have potent anti-oxidant activity. The *n*-butanol extract shows that it can act as a radical chain terminator that converts reactive free radicals to stable and non-reactive products. This is the origin of their capability to prevent lipid peroxidation chain reactions by donating a hydrogen atom from their

·OH groups. In addition, it was reported that phenolic and flavonoid contents are related to anti-oxidant capacity and have a necessary role in stabilizing for the lipid peroxidation (Koldaş et al., 2015).

The literature reported that the reductive ability of products is commonly related with the existence of reductants like anti-oxidant constituents (Özen, 2010). The great reducing capacity of extract may be the result of its effective amount of phenols and flavonoid compounds. Like relations concerning Fe³⁺-reducing ability and polyphenols have been declared in the literature (Gao et al., 2000).

Clearly, the ability of our plant to scavenge hydrogen peroxide could be due to the presence of flavonoid compounds and phenols. Similar studies suggested that the elimination of hydrogen peroxide is very necessary for the prevention of the living process (Koldaş et al., 2015).

The free radical scavenging activity was also increased with increasing extracts concentrations. Our data clearly indicated that the *n*-butanol extract of *P. coronopifolia* is a powerful free radical inhibitor. This result can consider to be noteworthy when compared to the finding of Boussaha et al. (2015) which suggests that this anti-oxidant ability can be attributed to extract's content of flavonoids like taxifolin, rhamnazin and caffeoylquinic acid derivatives determined in this plant.

The formation of ferrozine-Fe²⁺ complex is disrupted in the presence of *n*-butanol extract which indicates that it chelates iron. The metal chelating capacity of *n*-butanol extract was increased by increasing concentration. It was indicated that chelating compounds making α -bands with a metal are lesser anti-oxidants owing to reduction of the redox potential. It was reported in the literature that iron can provoke lipid peroxidation by Fenton-reaction, as can quicken it by decomposition of lipid hydroperoxides to peroxy (LO₂) and alkoxy (LO) radicals which may be able to capture hydrogen and propagate the formation of lipid peroxidation (Gutteridge, 1984).

ROS are known to have numerous damaging effects on the cells of the body. They severely induce lesions in DNA as deletions, mutations and genetic effects. Natural products in medicinal plants can scavenge the ROS generated by severing oxidative stress (Guha et al., 2011). Diverse study findings suggest that some plant extracts can protect DNA from oxidative damage by their anti-oxidant compounds (Arora et al., 2017). According to the reports, plant extract was practical to prevent DNA from oxidative injury induced by UV-photolysis of H₂O₂ (Lassed et al., 2015).

The current study was based on the ability of the *n*-butanol extract of *P. coronopifolia* to protect 46966 plasmid DNA against UV-photolysis of H₂O₂-induced

oxidative damage. Exposure of plasmid to UV-photolysis of H₂O₂ results in generation of hydroxyl radicals (OH). These radicals are known to cause oxidatively breakage of DNA strands, fragmentation of deoxy-sugars, modification of bases, oxidation of lipids and generation of end products like malondialdehydes and other unsaturated aldehydes, which can bind to DNA and result in mutagenic adducts (Chaudhary et al., 1994). According to the cumulative level of DPPH-scavenging, anti-oxidant activity, reducing power, hydrogen peroxide scavenging and metal chelating assays, the extract may provide DNA protection.

The electrophoretic pattern of 46966 plasmid-DNA using UV-photolysis of H₂O₂ in the absence or presence of extract. At a dose of 50 μ g the extract exhibited a complete protection of plasmid DNA, which indicates that the plasmid-DNA damaging is caused by hydroxyl radicals. The upon-treatment with *n*-butanol extract of *P. coronopifolia* protects the plasmid DNA from the hydroxyl radicals generated by UV-photolysis of H₂O₂. This investigation exhibits a noteworthy correlation between anti-oxidant potential and DNA protection. These results suggest that DNA loss inhibition may possibly owe to the great anti-oxidant capacity of the *n*-butanol extract of *P. coronopifolia*.

As compared with the positive control, the extract exposed varied profiles at different concentrations. HeLa cells were inactivated by *n*-butanol extract and showed the maximum activity at the concentration of 250 μ g/mL. However, C6 cells recovered from the inhibition in few hours. This possibly due to the lower concentration of active biomolecules in the plant extract, then cells initiated to proliferate after a few hours of inhibition.

The activity of higher concentration might be due to the chemical profiles as the high conception of polyphenolic and flavonoid content (Yaglioglu et al., 2013). While the diminution of this capacity might cause by the low bioactive molecule content of the plant extract which degraded within the time, cells can continue to grow and reproduce after inactivation. This *n*-butanol extract-findings agreed with the results of another study which suggests that the ethyl acetate extract of the same plant has an anti-oxidant potent and can inhibit the proliferation of HeLa and C6-cell lines at high concentrations with no cytotoxic effect observed (Boussaha et al., 2015).

Conclusion

The *n*-butanol extract of *P. coronopifolia* has possessed a positive DNA-damage protection ability, offered by its significant anti-oxidant potential. As compared with 5-fluorouracil, the findings suggest that our extract has a positive anti-cancer activity against HeLa cells at high

concentration.

Acknowledgement

This study was supported by the grants from the Algerian Ministry of Higher Education, Turkish State Planning Organization (DPT2010K120720), Çankırı Karatekin University (BAP Project: FF080515B30) and Ondokuz Mayıs University (BAP Project: PYO.FEN.1904.13.003).

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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