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Free Radical Scavenging Activity, Reducing Power and Anti-Hemolytic Capacity of Algerian *Drimia maritima* Baker Flower Extracts

Abir Rezzagui [1,2] *, Abderrahmane Senator[1,3], Soumia Benbrinis [1], and Hamama Bouriche [1]

[1] Laboratory of Applied Biochemistry, Faculty of Natural and Life Sciences, University Ferhat Abbas, Sétif 1, Sétif 19000, Algeria

[2] Department of Molecular and Cellular Biology, Faculty of Natural and Life Sciences, University of Jijel, Jijel 18000, Algeria

[3] Faculty of Natural and Life Sciences, University of Batna 2, Batna 5000, Algeria

ABSTRACT

This study was undertaken to evaluate the antioxidant and anti-hemolytic properties of Algerian *Drimia maritima* Baker flower extracts. Determination of phenolic content was carried out to estimate the chemical composition of *D. maritima* extracts. Antioxidant properties were investigated in all extracts using free radical scavenging activity (against DPPH, ABTS, hydroxyl radical, and superoxide anion), reducing power, inhibition of lipid peroxidation, and anti-hemolytic capacity. Phenolic determination revealed that *D. maritima* flowers contain phenolic compounds, flavonoids, and tannins. Ethyl acetate extract showed the highest reducing power and scavenging activity using DPPH and ABTS assays. However, aqueous extract was the most effective against hydroxyl radical, superoxide anion, and lipid peroxidation. The half-time of hemolysis indicates that chloroform extract exhibited the best anti-hemolytic capacity in the AAPH induced hemolysis model. The results of this study suggest that *D. maritima* could be used as a possible source of antioxidant phenolic compounds and that further determination of these compounds may provide more information on their medicinal value.

Keywords: *Drimia maritima*, phenolic compounds, scavenging activity, reducing power, anti-hemolytic.

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***Address for Correspondence:**

Abir Rezzagui, Laboratory of Applied Biochemistry, Faculty of Natural and Life Sciences, University Ferhat Abbas, Sétif 1, Sétif 19000, Algeria

INTRODUCTION

For many years, medicinal plants have been considered as a very important source of chemical substances with therapeutic effects. Thus, study of plants spices with traditional use as pain killers should be sees as a logical strategy in search for new drugs. *Drimia maritima* (*D. maritima*) is a wild-growing bulbous plant belonging to the family *Asparagaceae*. It is mainly distributed in slopes of hills and sandy soils of the Mediterranean Sea and North Africa[1]. In folk medicine, the bulb of this plant is used for the treatment of heart failure, chronic bronchitis, asthma, pneumonia, wounds, hemorrhoids, bites of vipers, jaundice and as a diuretic[2,3]. It is also used to treat dermatological problems, ear pain[4], digestive disorders[5], whooping cough[6] and hepatitis[7].

Several beneficial effects of *D. maritima* are attributed to the presence of many bioactive compounds. The most known are anthocyanins, flavonoids[8], fatty acids, polysaccharides[9], tannins, reducing compounds, anthraquinones, triterpenes, steroids[10] and cardiac glycosides (bufadienolids type) that are the major compounds with 1-3% of the bulb dry weight[11].

However, to our knowledge, no report is available on phenolic content and antioxidant activity of *D. maritima* flowers. So, the aim of the present study is to evaluate the radical scavenging activity and anti-hemolytic property of *D. maritima* flower extracts and their phenolic fractions, to understand the usefulness of this plant in traditional medicine.

MATERIAL AND METHODS

Plant material

Fresh *D. maritima* flowers were collected from Mila (east of Algeria), during the flowering season (October 2017). The plant material was air-dried in dark at room temperature and then ground to a powder using a manual grinder.

Preparation of extracts

The crud ethanolic extract (CRE) was prepared by macerating 100g of flower powder in 1000 mL of 80% ethanol with occasional shaking and the resulting macerate was filtered. These steps were repeated another time with the 50% ethanol. The resulting filtrate was evaporated under reduced pressure at 45°C. The filtrate was then washed with hexane several times until a clear upper layer of hexane was obtained. The lower layer was then extracted successively with chloroform and ethyl acetate to obtain at the end of the extraction process 2 fractions; chloroform fraction (CHE) and ethyl acetate fraction (EAE). Then solvents were removed using a rotary evaporator.

Aqueous extract (AQE) was prepared using an infusion of 100g of flower powder in 1000mL of boiling distilled water for 15min. After filtration and evaporation, the yield of each extract and fraction was determined before kept them in 4ºC until use.

Determination of phenolic content

Total phenolic content

The Folin-Ciocalteu reagent was used to determine total phenolic content in extracts[12]. A 100µL of samples at different concentrations (1, 2.5, 5mg/ml) was mixed with 500µL of Folin-Ciocalteau reagent (10% in distilled water). After 4min, 400µL of 7.5% of Na_2CO_3 solution were added. After incubation for 90min at room temperature, the absorbance was measured at 760nm and the results were expressed in mg of gallic acid equivalent per gram of extract (GAE/g).

Total flavonoid content

A volume of each sample (1, 2.5, and 5mg/ml) was mixed with an equal volume of aluminum chloride (2% in methanol) solution[13]. After incubation for 10min, the absorbance was measured at 430nm. All values were expressed as mg of quercetin equivalent per gram of extract (QE/g).

Total tannin content

The tannin content was determined according to the method described by Batesmith[14]. Briefly, 1mL of each extract at different concentrations (1, 2.5, 5mg/ml) was mixed with 1mL of hemolyzed bovine blood (absorbance = 1.6). After 20min, the mixture was centrifuged at 4000rpm for 10min and the absorbance of the supernatant was measured at 756nm. The results were expressed in mg equivalent of tannic acid per gram of extract (mg TAE/g).

Antioxidant activity

DPPH assay

A 500µL of different concentrations (0 – 350µg/ml) of the extracts were added to 500µL of DPPH solution (0.078mg/mL). After incubation for 30min at room temperature, the absorbance was measured at 517nm. BHT was used as a standard. The ability of extracts to scavenge DPPH radicals was calculated by the following equation[15]:

$$\text{DPPH radical scavenging activity (\%)} = (A_C - A_S / A_C) \times 100$$

A_C : absorbance without sample,
A_S : absorbance in the presence of the sample.

ABTS assay

ABTS cationic radical ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution (7mM) with 2.45mM potassium persulfate and allowing the mixture to stand at room temperature in the dark for 12–16 hours before use[16]. Then, the solution was diluted with methanol and equilibrated to give an absorbance of 0.700 ± 0.02 at 734nm. The scavenging activity was estimated by mixing 50µL of extracts at different concentrations (0–50µg/ml) or BHT with 1mL of $ABTS^{\bullet+}$ solution and absorbance was measured after 30min of incubation at room temperature. The antioxidant activity was calculated as the following equation:

$$ABTS^{\bullet+} \text{ scavenging activity (\%)} = (A_C - A_S / A_C) \times 100$$

A_C: absorbance without sample.

A_S: absorbance in the presence of the sample.

Superoxide anion scavenging activity

The superoxide anion scavenging activity was measured as described by Robak and Gryglewski[17]. These radicals are generated in 3mL of Tris–HCl buffer (16mM, pH 8.0), containing 500µL of nitroblue tetrazolium (NBT) (0.3mM), 500µL NADH (0.936mM) solution, 1mL of extract at different concentrations (0–1000µg/ml) and 500µL Tris–HCl buffer (16mM, pH 8.0). The reaction was initiated by adding a 500µL of phenazine methosulfate (PMS) solution (0.12mM) to the mixture. After incubation at 25°C for 5min, the absorbance was measured at 560nm. The antioxidant activity was calculated as the following equation:

$$\text{Superoxide anion radical scavenging activity (\%)} = (A_C - A_S / A_C) \times 100.$$

A_C: absorbance without sample.
A_S: absorbance in the presence of the sample.

Hydroxyl radical scavenging activity

The scavenging ability of hydroxyl radicals was measured according to the method of Smirnoff and Cumbes[18]. The reaction mixture consists of 1mL of ferrous sulfate (1.5mM), 700µL of hydrogen peroxide (6mM), 300µL of sodium salicylate (20mM), and 1mL of extracts at different concentrations (0–500µg/ml) or ascorbic acid. After incubation at 37°C for 1 hour and cooling, the absorbance was measured at 562nm. The antioxidant activity was calculated as the following equation:

$$\text{Hydroxyl radical scavenging activity (\%)} = [1 - (A_S - A_B / A_C)] \times 100$$

A_C: absorbance without sample,
A_B: absorbance of the blank (without sodium salicylate),
A_S: absorbance in the presence of the sample.

Reducing power

The ferric reducing capacity of the extracts was determined according to the method described by Prasad *et al.*[19]. So, 2.5mL of 0.2M phosphate buffer (pH 6.6) and 2.5mL of $K_3Fe(CN)_6$ (1%) were added to 1mL of sample at different concentrations (0-95µg/ml) dissolved in distilled water. The resulting mixture was incubated at 50°C for 20min, followed by the addition of 2.5mL of trichloroacetic acid (10%). The mixture was centrifuged at 3000rpm for 10min to collect the upper layer of the solution (2.5mL), which was mixed with 2.5mL of distilled water and 500µL of $FeCl_3$ (0.1%). The absorbance was then measured at 700nm. The Reducing power was represented as EC_{50} (effective concentration of the sample necessary to obtain 0.5 absorbance at 700nm).

β-carotene / linoleic acid essay

The ability of extracts to prevent bleaching of β-carotene was assessed as described by Kartal *et al.*[20]. A stock emulsion of β-carotene/linoleic acid was freshly prepared by dissolving 0.5mg of β-carotene in 1mL of chloroform, 25µl of linoleic acid, and 200mg of Tween 40. The chloroform was completely evaporated under vacuum in a rotatory

evaporator at 40ºC, then 100mL of distilled water was added and the resulting mixture was vigorously stirred. Aliquots (2.5mL) of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 500µL of each sample (400µg/ml) and the absorbance was measured at 470nm. The antioxidant activity was calculated as the following equation:

$$\text{Antioxidant activity (\%)} = (A_t\,(\text{extract}) / A_t\,(\text{BHT})) \times 100$$

A_t (extract): absorbance in the presence of extract at a specific time.
A_t (BHT): absorbance in the presence of the BHT at the same time.

Anti-hemolytic activity

The inhibition of AAPH-induced hemolysis in red blood cells (RBC) was determined according to Takebayashi *et al.*[21]. Briefly, AAPH (2.2-azobis (2-amidinopropane) dihydrochloride) dissolved in phosphate buffer solution (PBS, pH 7.4) was used to induce the oxidation chain in erythrocytes. Blood from a rat was collected in a tube containing 0.1% EDTA, centrifuged at 6000rpm for 10min and washed three times with PBS. Then, 100µL of the resulting red blood cells were immediately diluted to 2% by adding 4.9mL of PBS. To assess the anti-hemolytic activity of extracts, 120µL of the blood solution was pre-incubated at 37°C, for 15min, with 60µL of extracts or Trolox (12.5, 25 and 50µg/ml). After that, 120µL of AAPH solution (300mM) was added and the reaction mixtures were then incubated at 37°C for 4-5 hours. The degree of hemolysis was determined every 30min at 620nm using a 96-well micro-plate reader (ELX 800 de Bio-TEK instruments). The RBC hemolysis was calculated as the following equation:

$$\text{RBC hemolysis (\%)} = (A_0 - A_X / A_0 - A_{Final}) \times 100$$

A_0: absorbance at the start of the experience.
A_X: absorbance at a specific time.
A_{Final}: absorbance at the end of the experience.

RBC resistance to the AAPH-induced hemolysis is estimated using the half time of hemolysis (HT_{50}) which corresponds to the necessary time to induce hemolysis of 50% of initial erythrocytes.

Statistical analysis

Results were expressed as means of triplicate ± SD. Data were statistically analyzed with Graph Pad Prism® version 8.0.1, using one-way analysis of variance (ANOVA) and samples differences were extrapolated by Tukey's multiple comparison test. Differences are considered significant when $P < 0.05$.

RESULTS

Extraction yield, total phenolic, flavonoid and tannin content

Table 1 presents the results obtained for yield, total phenolic, flavonoid, and tannin content of *D. maritima* extracts. The results revealed that higher levels of total phenolics, flavonoids, and tannins were observed in EAE compared to the other extracts (Table 1).

Table 1: Yield, total phenolic, flavonoid and tannin content of *D. maritima* extracts

Extract	Yield (%)	Total phenolic (mg of GAE/g of extract)	Total flavonoid (mg of QE/g of extract)	Total tannin (mg of TAE/g of extract)
AQE	20.4	68.7 ± 3.1	3.45 ± 0.1	391.1 ± 17.3
CRE	24.9	66.9 ± 1.4	5.31 ± 0.03	314.4 ± 3.3
EAE	3	100.4 ± 1.3	29.19 ± 0.2	471.8 ± 0.2
CHE	1.7	73.6 ± 3.2	9.49 ± 0.2	422.7± 3.5

Values are means of triplicate determinations (n=3) ± SD.

Antioxidant activity

DPPH radical scavenging activity

Data presented in Figure 1 showed that various extracts of *D. maritima*, at different concentrations, exerted scavenging activity against DPPH radicals in a concentration-dependent manner. Among all extracts, EAE and AQE were the most effective extracts with the lowest IC_{50} values. These values are close to those obtained with BHT (Table 2).

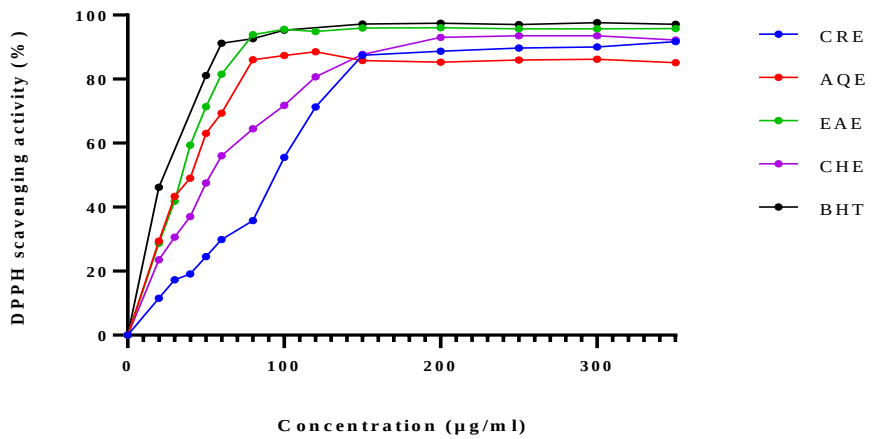

Figure 1: DPPH radical scavenging activity of *D. maritima* extracts and BHT. Values are means of triplicate determinations (n=3) ± SD.

Table 2: IC_{50} and EC_{50} of *D. maritima* extracts in ABTS, reducing power, DPPH, anion superoxide, and hydroxyl radical assays.

Samples	DPPH (IC_{50}, µg/mL)	ABTS (IC_{50}, µg/mL)	Hydroxyl radical (IC_{50}, µg/mL)	Anion superoxide (IC_{50}, µg/mL)	Reducing power (EC_{50}, µg/mL)
CRE	94.66 ± 1.75***	25.77 ± 0.69***	406.67 ± 1.4***	445.77 ± 30.23***	53.12 ± 0.017***
AQE	40.88 ± 4.03 ns	26.33 ± 0.52***	189.17 ± 0.68***	147.78 ± 1.72***	50.56 ± 0.011***
EAE	34.22 ± 1.86 ns	4.99 ± 0.55**	239.17 ± 1.71***	202.77 ± 9.22***	17.06 ± 0.014***
CHE	54.66 ± 2.42***	7.33 ± 0.62***	ND	562.78 ± 13.72***	24.2 ± 0.006***
BHT	33.99 ± 4.42	1.66 ± 0.26	--------	-------	4.393 ± 0.014
Vit C	-------	-------	75 ± 2.24	7.04 ± 1.46	-------

Values are means of triplicate determinations (n=3) ± SD. ND: not determined. **P<0.01; *** P<0.001; ns: not significant compared to BHT or Vitamin C.

ABTS radical cation scavenging activity

As well as DPPH, ABTS scavenging activity of all extracts was concentration-dependent (Figure 2). The IC_{50} values of different extracts were ranged from 4.99 ± 0.55µg/mL for EAE to 26.33 ± 0.52µg/mL for AQE (Table 2). Again, EAE exhibited the highest antioxidant activity compared to other extracts. This activity was similar to that obtained with BHT. The correlation analysis indicated the presence of a good relationship between phenolic compounds and ABTS antioxidant activity of the extracts of *D. maritima* (r = 0.779 for total phenolic, r = 0.808 for flavonoid and r = 0.847 for tannin).

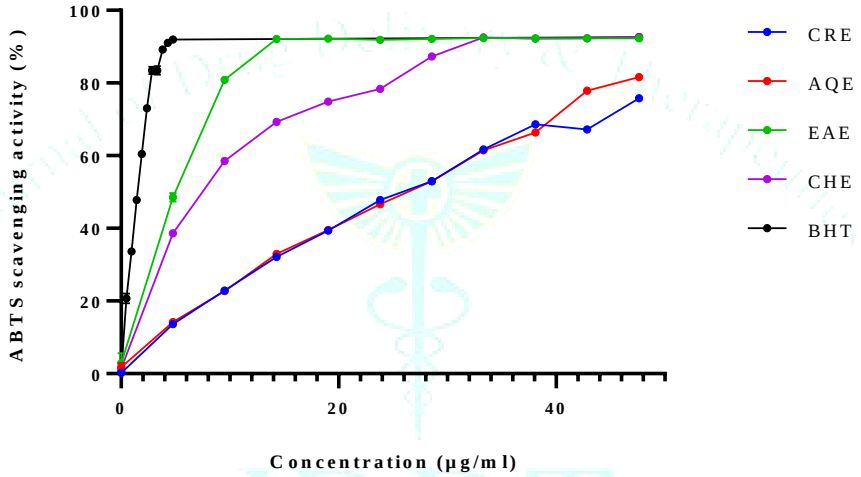

Figure 2: ABTS radical scavenging activity of *D. maritima* extracts and BHT. Values are means of triplicate determinations (n=3) ± SD.

Superoxide anion scavenging activity

The studied extracts exerted a superoxide scavenging activity with a concentration-dependent manner (Figure 3). Table 2 showed that AQE was the most effective extract with the lowest IC_{50}, but this value was significantly ($P < 0.001$) higher than that of vitamin C.

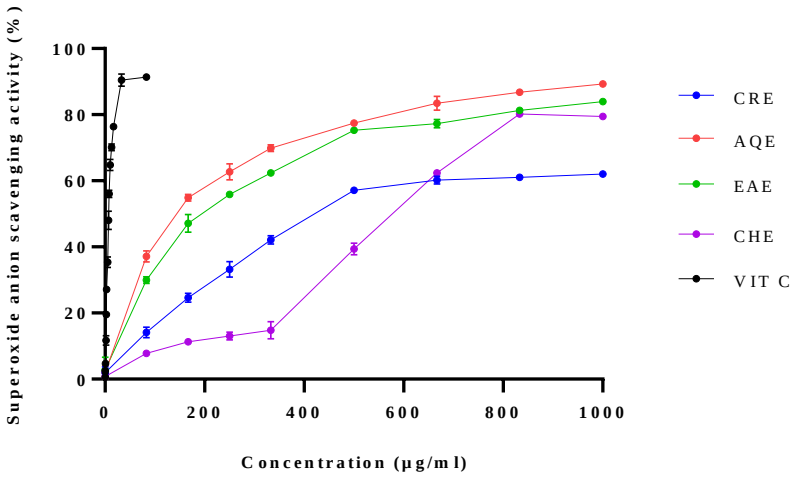

Figure 3: Superoxide anion scavenging activity of *D. maritima* extracts and vitamin C. Values are means of triplicate determinations (n=3) ± SD.

Hydroxyl radical scavenging activity

Figure 4 showed that all extracts and vitamin C can scavenge hydroxyl radicals. Among *D. maritima* extracts, AQE was the strongest radical scavenger followed by EAE (Table 2). The hydroxyl radical scavenging ability was in descending order (Vitamin C > AQE > EAE > CRE > CHE).

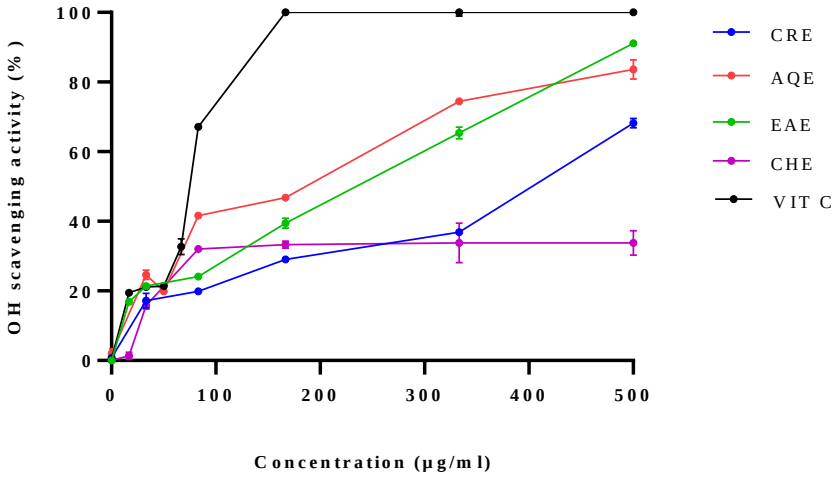

Figure 4: Hydroxyl radical scavenging activity of *D. maritima* extracts and Vitamin C. Values are means of triplicate determinations (n=3) ± SD.

Reducing power

All the extracts exhibited a ferric reducing capacity in a concentration-dependent manner (Figure 5). A strong activity was exerted by EAE (Table 2), but this activity was lower than that of BHT. The reducing power of samples was in the following order: BHT > EAE > CHE > AQE > CRE. As well as ABTS, the results showed a significant correlation between phenolic compounds in *D. maritima* extracts and their reducing power (r =0.787 for total phenolic, r = 0.804 for total flavonoid and r = 0.811 for total tannin).

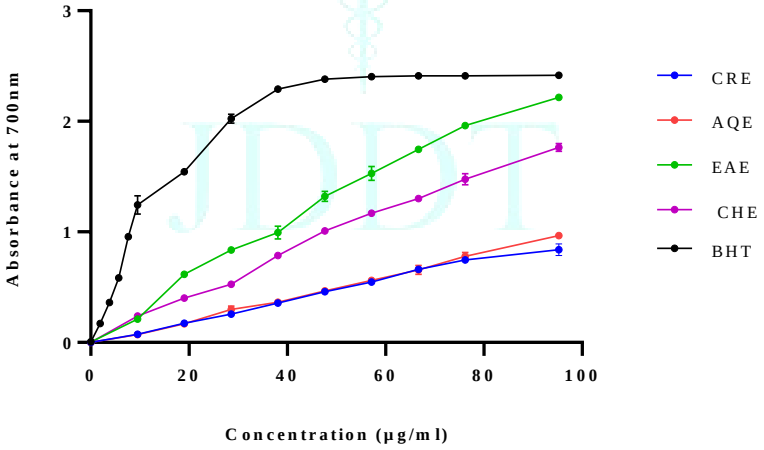

Figure 5: Reducing power of *D. maritima* extracts and BHT. Values are means of triplicate determinations (n=3) ± SD.

Inhibition of β-carotene bleaching

Figure 6 showed that all extracts inhibit β-carotene oxidation and bleaching for 48hours. AQE exerted the strongest inhibition on β-carotene oxidation (71%) followed by CHE (56%), EAE (30%), and CRE (20%).

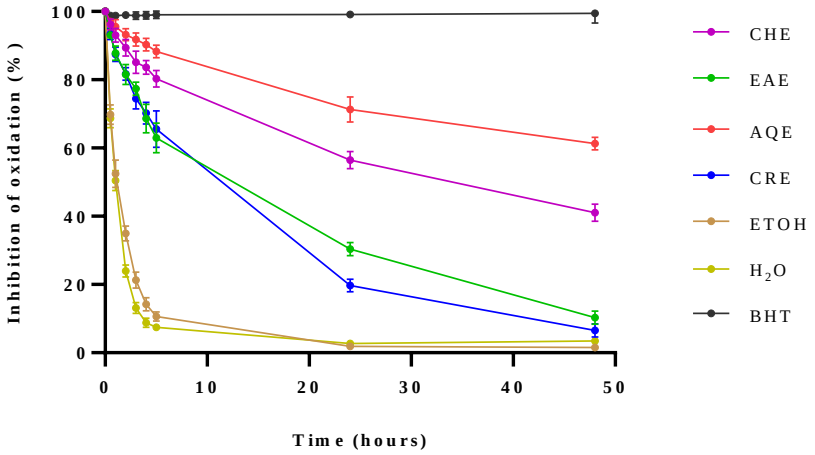

Figure 6: Kinetic of the inhibition of linoleic acid/β-carotene oxidation by *D. maritima* extracts and BHT. Values are means of triplicate determinations (n=3) ± SD.

Anti-hemolytic activity

The addition of AAPH to the blood solution induced erythrocytes hemolysis after 125min of incubation, leading to a maximum hemolysis at 220min (Figure 7). However, the pre-incubation of erythrocytes with different concentrations of extracts or Trolox attenuated significantly AAPH-induced erythrocyte hemolysis in a concentration- and time-dependent manner.

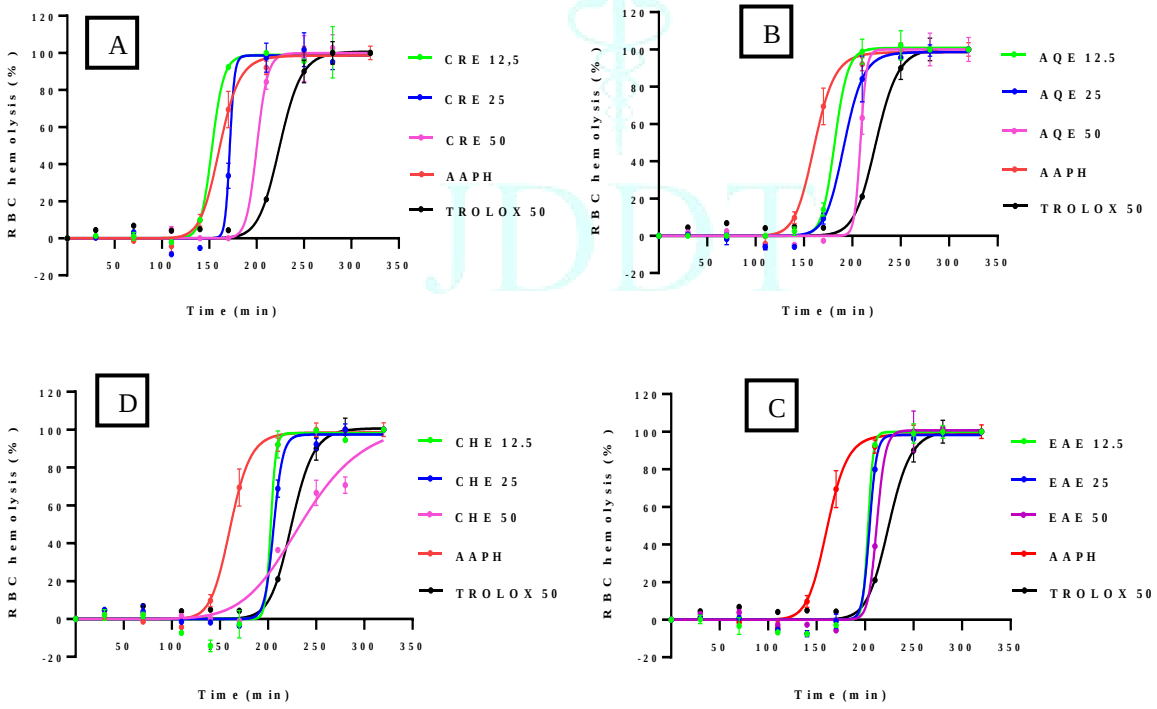

Figure 7: Inhibition of red blood cells hemolysis by *D. maritima* extracts and Trolox. Values are means of triplicate determinations (n=3) ± SD.

Figure 8 indicated that all extracts exhibited a high protective effect against RBC hemolysis and increased significantly the half time hemolysis compared to AAPH alone. Among the extracts, CHE was the most effective (half time of hemolysis = 223.4min).

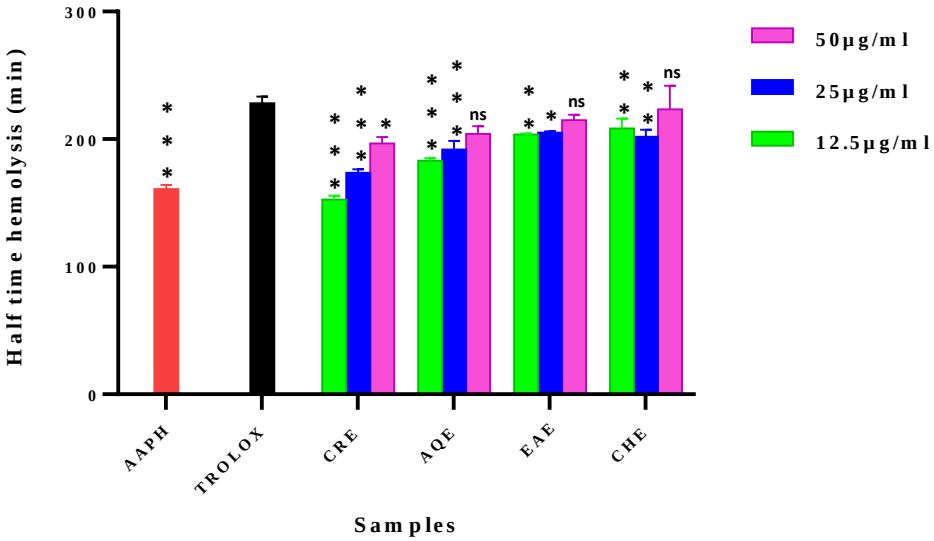

Figure 8: Half-time hemolysis of RBC by *D. maritima* extracts (12.5, 25, 50µg/ml). Values are means of triplicate (n=3) ± SD. *P<0.05; **P<0.01; ***<0.001; ns: not significant compared to Trolox (50µg/ml).

DISCUSSION

The present study revealed that extracts of *D. maritima* flowers are relatively rich in phenolic compounds, especially tannins. Similar studies have indicated that tubers and leaves of *D. maritima* are rich in phenolic compounds[22, 23].

Radical scavenging and antioxidant properties of plant extracts are associated with their phytochemical content[24]. However, using a single assay to estimate the antioxidant properties may be insufficient, because it could be influenced by many factors. For this reason, it is very important to carry out more than one type of antioxidant assay to cover the different mechanisms of the antioxidant activity[25]. In this study, DPPH, ABTS, hydroxyl radical and superoxide anion assays were used to estimate the radical scavenging properties of *D. maritima*.

DPPH radical scavenging activity is an easy, rapid, and sensitive way to study the antioxidant activity[26]. DPPH• is a stable free radical, whose color changes from violet to yellow after reduction by hydrogen donating antioxidants of plant extracts[27]. Otherwise, $ABTS^{\bullet+}$ is relatively stable but readily reduced by antioxidants leading to its decolorization. This assay is an excellent tool to determine the antioxidant activity of hydrogen-donating antioxidants and chain-breaking antioxidants[16]. *D. maritima* exhibits a scavenging activity against both DPPH and ABTS radicals. This ability may be due to the presence of phenolic compounds especially tannins. Hagerman *et al.*[28] have reported that the high molecular weight phenolic compounds such as tannins have more ability to quench free radicals ($ABTS^{\bullet+}$). This effectiveness depends on the molecular weight, the number of aromatic rings, and the nature of hydroxyl group substitution. These facts could explain the presence of a high correlation between $ABTS^{\bullet+}$ radical scavenging and tannins content in *D. maritima* extracts. The results of this study are following other studies that indicated that extracts of *D. maritima* bulbs and leaves or *D. indica* bulbs are good radical scavengers[22, 29].

Hydroxyl radical is the most reactive free radical formed in biological systems and it can damage almost every molecule in living cells, such as fatty acids, proteins, and DNA inducing severe cellular damages[30]. Therefore, the removal of hydroxyl radicals can protect humans against several diseases[31]. Although superoxide anion is a weak oxidant, it ultimately produces powerful and dangerous hydroxyl radicals, which contributes to oxidative stress[32]. Results revealed that ethyl acetate extract and chloroform extract of *D. maritima* displayed different potential in scavenging hydroxyl radical and superoxide anions. This potential is positively associated with the amount of total phenolic compounds in the extracts, which have active hydrogen donor ability of hydroxyl substitution[33].

Reducing power is used as a significant reflection of the antioxidant activity. The reducing property is generally attributed to the presence of reductants. The antioxidant action of reductants is based on the breaking of the free radical chain by electron donation. Moreover, reductants react with the precursors of peroxide, thus preventing peroxide formation[34]. The results presented in this study indicated the presence of a very good correlation between tannin contents and the reducing power of *D. maritima* extracts. According to this finding, it seems that reducing the activity of *D. maritima* extracts is due to the presence of phenolics such as tannins. Indeed, these compounds may act as reductants by donating the electrons and reacting with free radicals to convert them to more stable products and terminate radical chain reaction[27]. A relationship between the phenolic constituents and reducing power activity has been reported for several plant extracts[27,35].

In the β-carotene/linoleic acid system, the oxidation of linoleic acid generates peroxyl free radicals due to the abstraction of a hydrogen atom from diallylic methylene groups of linoleic acid[36]. The generated peroxyl radicals will oxidize the highly unsaturated molecule of β-carotene. However, the presence of antioxidants in the extract could minimize the β-carotene oxidation[13]. This inhibition may be

due to the hydrogen donating capacity of phenolic compounds. Indeed, several studies that focused on medicinal plants, fruits, or vegetables found a high correlation between phenolic content of extracts and antioxidant activity in the β-carotene/linoleic acid system[27,37].

The decomposition of the water-soluble AAPH at physiological temperature generates *in vitro* free radicals that attack erythrocytes membranes and induce lipid peroxidation, leading to hemolysis. This method is very useful for screening activities of various molecules, especially those having an oxidant or antioxidant activity with a long-term action[27]. Natural antioxidants like phenolic compounds can scavenge and react with free radicals and then terminate the free radical reaction chain. Hence, the suppression of the oxidative modification of erythrocyte lipids by antioxidants constitutes the preferable strategy to prevent hemolysis and cardiovascular diseases[38].

The pretreatment by *D. maritima* extracts increased significantly the resistance of erythrocytes against hemolysis. It has been reported that the pretreatment with an antioxidant increases the half-time of hemolysis, which links to a good resistance of erythrocytes[27]. This anti-hemolytic activity could be explained by the inhibition and neutralization of free radicals liberated by AAPH decomposition. Such hypothesis could be supported by the presence of a good relationship between the percentage of hemolysis and reducing power (r = 0.663), $ABTS^{\bullet+}$ (r = 0.808) and tannins (r = 0.838), suggesting that the mechanism of action of the extracts of *D. maritima* flowers against hemolysis may be related to their content in tannins, reducing power and radical scavenging ability. Several investigators have established the presence of a correlation between phenolic compounds and anti-hemolytic activity in plant extracts[27,39].

CONCLUSION

Our finding increases the interest in the use of *D. maritima* flowers as a source of pharmacological agents. Extracts of *D. maritima* flowers contain high levels of total phenolic compounds, especially tannins and they were able to scavenge free radicals and thereby terminate the radical chain reactions. Further determination of compounds from this plant and the study of other biological effects may provide more information on their medicinal value.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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