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In vitro antioxidant capacity of *Euphorbia retusa* Forssk. from Algerian desert

[Capacidad antioxidante *in vitro* de *Euphorbia retusa* Forssk. proveniente del desierto argelino]

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Abstract

Context: *Euphorbia retusa* Forssk. is a medicinal plant, which traditionally is used in Algerian Sahara for treatment of infantile eczema, warts and trichiasis.

Aims: To evaluate phenolic composition and antioxidant capacity of methanolic extracts from stems, leaves and seeds of *Euphorbia retusa*.

Methods: Total phenolic and flavonoids were analyzed by Folin-Ciocalteu and aluminum chloride assays, respectively using gallic acid (GA) and quercetin (Q) as standard compounds. Antioxidant capacity was studied *in vitro* by using DPPH and ABTS cation radical scavenging, cupric-reducing antioxidant capacity (CUPRAC) and reducing power assays, followed by identification of phenolic compounds of seeds by mean of high-performance liquid chromatography (HPLC-DAD).

Results: Methanolic extracts of *E. retusa* seeds had the highest total phenolic and flavonoid contents with 315.50 ± 1.41 µg GA equivalent/mg dry extract and 91.83 ± 8.18 µg Q equivalent/mg dry extract, respectively. Identification of phenolic compounds from seeds revealed that epigallocatechin 3-O- gallate was the major flavonoid. This organ showed the highest activity in the assays of DPPH (IC_{50} = 7.20 ± 0.25 µg/mL), ABTS (IC_{50} < 6.25 µg/mL), CUPRAC ($A_{0.5}$ = 10.64 ± 0.99 µg/mL) and reducing power ($A_{0.5}$ = 11.84 ± 1.72 µg/mL).

Conclusions: *Euphorbia retusa* seeds have an antioxidant capacity that could be useful in pharmacological and medicinal fields.

Keywords: antioxidant; *Euphorbia retusa*; HPLC-DAD; phenolics.

Resumen

Contexto: *Euphorbia retusa* Forssk. es una planta medicinal que tradicionalmente se utiliza en el Sahara argelino para el tratamiento del eccema infantil, verrugas y triquiasis.

Objetivos: Evaluar la composición fenólica y la capacidad antioxidante de los extractos metanólicos de tallos, hojas y semillas de *Euphorbia retusa*.

Métodos: Los compuestos fenólicos y flavonoides totales se analizaron mediante ensayos de Folin-Ciocalteu y cloruro de aluminio, respectivamente, utilizando ácido gálico (GA) y quercetina (Q) como compuestos estándares. La capacidad antioxidante se estudió *in vitro* mediante la eliminación de radicales catiónicos DPPH y ABTS, los ensayos de capacidad antioxidante reductora cúprica (CUPRAC) y de potencia reductora, seguido de la identificación de compuestos fenólicos de semillas mediante cromatografía líquida de alta resolución (HPLC-DAD).

Resultados: Los extractos metanólicos de las semillas de *E. retusa* tuvieron los contenidos más altos de fenoles y flavonoides totales con 315.50 ± 1.41 µg de GA equivalente/mg de extracto seco y 91.83 ± 8.18 µg de Q equivalente/mg de extracto seco, respectivamente. La identificación de compuestos fenólicos a partir de semillas reveló que la epigalocatequina 3-O-galato fue el principal flavonoide. Este órgano mostró la mayor capacidad antioxidante en los ensayos DPPH (IC_{50} = 7.20 ± 0.25 µg/mL), ABTS (IC_{50}<6.25 µg/mL), CUPRAC ($A_{0.5}$ = 10.64 ± 0.99 µg/mL) y poder reductor ($A_{0.5}$ = 11.84 ± 1.72 µg/mL.

Conclusiones: Las semillas de *Euphorbia retusa* tienen una capacidad antioxidante que podría ser útil en farmacología y medicina.

Palabras Clave: antioxidante; *Euphorbia retusa;* HPLC-DAD; fenoles.

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INTRODUCTION

The Sahara, which covers about third of the land surface, encompasses a relatively sparse community of wild plants, 1200 species as compared to their surface (Benchelah et al., 2000). However, flora desert as well as medicinal plants have developed an import resistance strategy for unfavorable extreme conditions of their habitat such as high temperature, salinity, drought and high light intensities. In this stage, plants respond to environmental stress to withstand and to eliminate toxic reactive oxygen species (ROS) by developing ROS-detoxification mechanisms that can be divided into enzymatic and non-enzymatic systems (Trabelsi et al., 2010).

Furthermore, phenolic compounds are known noteworthy for their capacity of acquiring resistance to the environmental stress. These compounds exhibit an essential role as antioxidants and as a buffer of redox potential (Macheix et al., 2005; Oueslati et al., 2012; Falleh et al., 2012).

In recent time, focus on natural antioxidants increased all over the world to replace synthetic antioxidants because of their undesirable side effects on human health (Trabelsi et al., 2010).

In the other hand, the ethnobotanical knowledge of the Sahara populations has been used since immemorial time, which is an important tool to facilitate research on the sources of antioxidants. Besides, medicinal plants in desert area have higher antioxidant proprieties of the phenolic compounds because of their high redox ability (Trabelsi et al., 2010; Gasmi et al., 2019).

Euphorbia retusa Forssk. (synonym *E. cornuta* Pers.) is known as traditional remedy of infantile eczema, warts and trichiasis in the central Sahara of Algeria (Hammiche and Maiza, 2006). In Saudi Arabia *E. retusa* is used as local herbal medicine and as anti-asthmatic and anti-cough (Abdallah, 2015). It is an annual plant, which grows naturally up to 30 cm high in the Sahara (Quezel and Santa, 1962). Likewise, the chemical constituents of Algerian *E. retusa* have been investigated only for terpenoids (Haba et al., 2009); even there are a few data about the antioxidant activities as well as phenolic composition of this species of *E. retusa* organs. So, the aim of this study was to determine the antioxidant capacity of methanolic extracts of *E. retusa* stems, leaves and seeds followed by identification of phenolics compounds in seeds extract using HPLC-DAD in order to improve the knowledge on this plant, to select the organ, which has a highest antioxidant capacity and to provide scientific support for its possible future development in the medicinal and pharmaceutical fields.

MATERIAL AND METHODS

Chemicals

Methanol, quercetin (Q), Folin–Ciocalteu's reagent (FCR), gallic acid, potassium acetate, aluminum nitrate, potassium persulfate, copper (II) chloride, sodium carbonate, potassium ferricyanide, phosphate buffer, trichloroacetic acid (TCA), ferric chloride, neocuproine, ammonium acetate, butylated hydroxyl anisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, 2,20-azinobis(3-ethyl-benzothiazoline- 6-suphonic acid) diammonium salt (ABTS), were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant material

E. retusa was collected in June 2016 from Biskra region (north-east of Algeria: 34.896000, 5.756000). Plant identification was confirmed by researcher Rabia Sahki-Boutammine, National Forest Research Institute of Algeria. A voucher specimen was kept in the herbarium of the Laboratory of Biosystematic, Scientific and Technical Research Center for Arid Areas (CRSTRA), Biskra, Algeria. After harvesting, different parts of the plant (stems, leaves and seeds) were separated, cleaned and dried under shade at room temperature for seven days. After drying, the samples were prepared as a powder by blending each part of organs.

Soxhlet extraction

Twenty grams (20 g) of powder of each part (stem, leave and seed) were placed in a Soxhlet

apparatus. Extraction was performed with 200 mL of methanol for 8 hours. After that, the solvent was evaporated under pressure in a rotary evaporator (R215, BÜCHI Labortechnik AG, Flawil, Switzerland) at 50°C to give a solid dry extract (DE).

Bioactive compounds

Total phenolic content

The total phenolic content in methanolic extracts was determined by using Folin-Ciocalteu according to the method of Singleton and Rossi (1965) with slight modification. The protocol was based on mixing 20 µL sample with 100 µL Folin–Ciocalteu reagent and 75 µL (7.5%) sodium carbonate. Absorbance was measured at 740 nm in the microplate reader after 2 h incubation in darkness at room temperature by using a 96-well microplate reader (Perkin Elmer, Enspire) (Amrani et al., 2019). The absorbance was measured at 765 nm by using a 96-well microplate multimode plate reader (EnSpire, PerkinElmer, MA, USA). The experiment was done in triplicate and the total phenolic compounds were expressed as gallic acid equivalents/mg DE (µg GAE/mg DE). Calibration equation for total phenol determination was found as $y = 0.002x + 0.010$ ($r^2 = 0.989$).

Total flavonoid content

Total flavonoids were determined according to Türkoğlu et al. (2007) with slight modification. The method is based on the complexation with aluminium (Al^{3+}). A 50 µL of the diluted extract solution was mixed with 1900 µL of methanol, 50 µL of 1 M potassium acetate and 50 µL of 10% aluminum nitrate. Then, the obtained mixture was distributed in the wells of 96-well microtiter plates in triplicate (200 µL for each extract mixture). After incubation at room temperature in dark for 40 min, the absorbance was measured at 415 nm by using a 96-well microplate reader (EnSpire, PerkinElmer, MA, USA). The experiment was done in triplicate and total flavonoid content was expressed as µg of quercetin equivalents per mg of dry extract (µg QE/mg DE) and calibration equation for the determination of total flavonoid contents was found as $y = 0.0066x - 0.0063$ ($r^2 = 0.998$).

Phenolics identification by HPLC

The identification of phenolic compounds was done using an HPLC system consisting of a vacuum degasser, an autosampler, and a binary pump with a maximum pressure of 400 bar (Agilent 1260, Agilent Technologies, Germany) equipped with a reversed phase C18 analytical column of 4.6 x 100 mm and 3.5 µm particle size (Zorbax Eclipse XDB C18). The DAD detector was set to a scanning range of 200 - 400 nm. Column temperature was maintained at 25°C. A solvent system with a gradient of solvent A (methanol) and solvent B (1% formic acid) was used. Seed methanolic extracts were hydrolyzed according to the slightly modified method of Proestos et al. (2006). The flowrate of the mobile phase was 0.4 mL/min. The optimized gradient elution was performed as follows: 0-5 min, 10-20% A; 5-10 min, 20-30% A; 10-15 min, 30-50% A; 15-20 min, 50-70% A; 20-25 min, 70-90% A; 25-30 min, 90-50% A; 30-35 min, return to initial conditions. The peaks were monitored at 280 nm and were identified according to the retention time of calibration of 37 standards (Annex 1).

Antioxidant capacity

DPPH free radical-scavenging capacity

The free radical scavenging capacity of methanolic extracts was evaluated using DPPH assay as described by Blois (1958). About 40 µL of the each methanolic extract concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL) was added to 160 µL of a methanolic DPPH solution (6 mg DPPH dissolved in 100 mg methanol). The mixture was shaken vigorously and left standing at room temperature in the dark for 30 min. The experiment was done in triplicate and the absorbance was determined at 517 nm by using a 96-well microplate reader. Butylated hydroxy anisole (BHA) was used as a standard and the ability to scavenge the DPPH radical of each extract concentrations was calculated using the following equation [1]:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1 / A_0)] \times 100 \quad [1]$$

Where A_0 was the absorbance of the negative control and A_1 was the absorbance of the sample at 30 min.

$ABTS^{\bullet+}$ scavenging capacity

ABTS radical-scavenging capacity of sample extracts was determined according to the method of Re et al. (1999). The $ABTS^{\bullet+}$ was obtained by mixing 19.2 mg of 7 mM ABTS solution and 3.3 mg of 2.45 mM potassium persulfate solution, this emulsion was stored for 16 h at room temperature in the dark. Subsequently, the $ABTS^{\bullet+}$ was diluted with ethanol to absorbance of 0.750 (± 0.020) at 734 nm. Then, 160 µL of $ABTS^{\bullet+}$ solution was added to 40 µL of sample solution in methanol at different concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL) and these mixtures were homogenized. The experiment was done in triplicate and the absorbances were recorded at 734 nm by using a 96-well microplate reader. After 10 min, the inhibition percentage of each concentration was calculated relative to a blank absorbance (methanol) following the equation [1]. BHA was used as antioxidant standard for comparison of the activity.

Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity of methanolic extracts was determined according to the method of Apak et al. (2004). To an Eppendorf, 50 µL each of 10 mM Cu (II), 50 µL neocuproine of 7.5 mM, and 60 µL ammonium acetate (1 M, pH 7.0) solutions were added. About 40 µL of extracts at different concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL) were transferred to the initial mixture. The experiment was done in triplicate and the obtained emulsion was distributed in the wells of 96-well microtiter plates. After 60 min, the absorbance of the emulsions was read at 450 nm by using a 96-well microplate reader. BHA was used as a positive control. Results were expressed as absorbance for each concentration against a reagent blank and $A_{0.50}$ values (µg/mL) corresponding the concentration indicating 0.50 absorbance intensity.

Reducing power

The reducing power of the tested compounds was determined according to the method of Bouratoua et al. (2017). In order to determine the reducing power capacity, 10 µL of serially diluted sample of each concentrations (1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL) were added into a 96 well round-bottomed plate. Following this, 40 µL of 0.2 M phosphate buffer (pH 6.6) and 50 µL of potassium ferricyanide (1%), were added to each well and the plate was incubated at 50°C for 20 min. Finally, 50 µL of tricarboxylic acid (10%), distilled water (40 µL) and 10 µL of ferric chloride (0.1%) were added into each well in order to measure the reducing power capacity. Ascorbic acid was used as an antioxidant standard. The experiments were done in triplicate and the absorbance was measured in a microplate reader at 700 nm. Results were expressed as absorbance for each concentration against a reagent blank and $A_{0.50}$ values (µg/mL) corresponding the concentration indicating 0.50 absorbance intensity.

Statistical analysis

Results were expressed as mean ± standard deviation of three replicates using the STATISTICA program. Analysis of one-way variance (ANOVA) was performed and significant differences among means were compared by Newman–Keuls Multiple Range test ($p<0.05$).

RESULTS

Bioactive compounds

Total phenolic and flavonoid contents

Methanolic seed extracts had more phenolic content as compared to stem and leaf extracts. (Table 1). Also, the flavonoid contents in the three organs ranged from 91.83 ± 8.18 to 14.06 ± 5.64 (µg QE/mg DE). It was higher in seeds extract. Then, flavonoids represented 29.11% of total polyphenol content in seeds and it almost similar rate in stems and leaves (16.67% and 17.86%, respectively).

Table 1. Total phenolic and flavonoid contents of the methanolic extracts of *E. retusa.*

Extracts	Total phenolic (µg GAE/mg DE)	Total flavonoid (µg QE/mg DE)
Stems	87.75 ± 9.44[b]	15.67 ± 3.79[b]
Leaves	84.33 ± 6.66[c]	14.06 ± 5.64[b]
Seeds	315.50 ± 1.41[a]	91.83 ± 8.18[a]

Values expressed as mean ± SD (n=3). In the same column followed by a different letter (a-c) are significantly different ($p<0.05$). µg GAE/mg DE: microgram gallic acid equivalent per milligram of dry plant extract. µg QE/mg DE: microgram quercetin equivalent per milligram of dry plant extract.

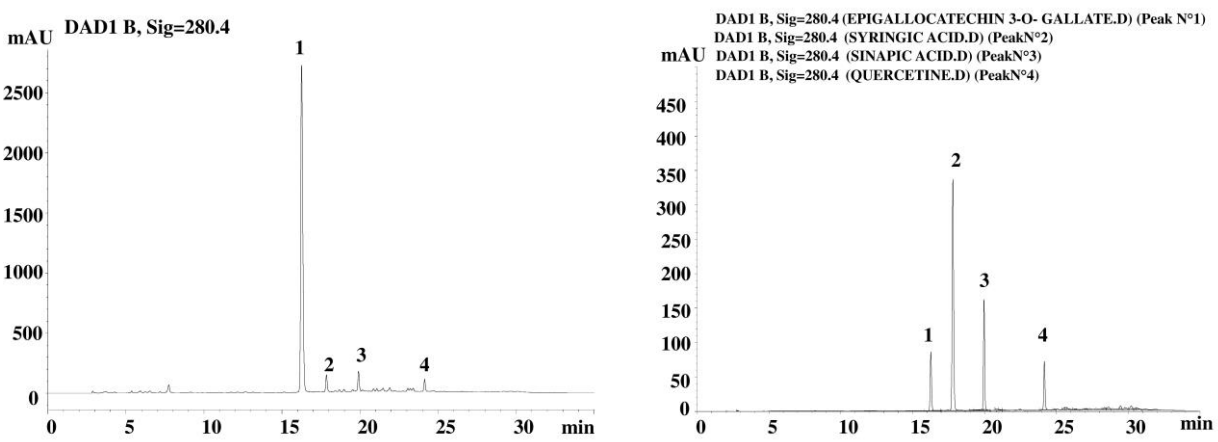

Figure 1. Chromatographic profiles acquired at 280 nm of seeds methanolic extract **(A)**, and standards mixture **(B)**.

The peak numbers correspond to **1.** epigallocatechin 3-O-gallate (Rt = 16.2 min); **2.** syringic acid (Rt = 17.8 min); **3.** sinapic acid (Rt=19.9 min); **4.** quercetin (Rt = 24.1 min).

Phenolics identification in E. retusa seeds by HPLC

Due to the highest antioxidant capacity and high concentration of phenolic compounds of *E. retusa* seeds, their crude extracts were retained for HPLC analysis (Fig. 1). The chromatogram of seeds extract was compared to authentic 37 standards of phenolic acids and flavonoids. Profiles composed by four identified phenolic compounds: epigallocatechin 3-O- gallate, syringic, sinapic acid and quercetin (Fig. 1A). These compounds have been identified according to their retention time and the spectral characteristics of their peaks compared to those of standards in Fig. 1B. Moreover, chromatogram shows that epigallocatechin 3-O-gallate was the major flavonoid compound in the seed extracts.

Antioxidant capacity

DPPH free radical scavenging capacity of stem, leaf and seed methanolic extracts is shown in Table 2. DPPH scavenging ability increased with an increase in concentration of extracts between 6.25 and 100 µg/mL and the inhibition percentage of DPPH radical in seed extracts was higher compared with BHA, stem and leaves. Similarly, low IC_{50} value represents a high potency and seed extracts had the highest DPPH radical scavenging capacity.

The experimental data of ABTS scavenging reduction (Table 3) indicated that the inhibition percentage of seed extracts was more than 60% at 6.25 µg/mL as well as BHA. Therefore, stem extracts

exhibited higher capacity to scavenging the $ABTS^{\bullet+}$ radical than leaf extracts between 25 and 400 µg/mL.

The results of CUPRAC test (Table 4) showed that seeds had a higher capacity than stem and leaf extracts. However, none of the extracts exhibited higher activity than standards BHA. Moreover, stem extracts exhibited higher capacity than leaf extracts.

Concerning reducing power capacity (Table 5), the absorbance of the extracts as well as the standard BHA increased with increasing of concentrations. The higher absorbance of the reaction mixture indicated a higher activity. Seed methanolic extracts had very high reducing power at all concentrations compared with stems and leaves extracts. Then, seeds extract indicates a low $A_{0.50}$ value as compared with ascorbic acid.

Table 2. Antioxidant capacity of the methanolic extracts of *E. retusa* by the DPPH scavenging assay.

Concentration (µg/mL)	**Inhibition** (%)			
	Stems	**Leaves**	**Seeds**	**BHA**
6.25	0.99 ± 1.16	-	44.63 ± 1.25	36.46 ± 2.45
12.5	4.08 ± 0.74	-	80.10 ± 2.37	59.63 ± 1.50
25	5.29 ± 1.96	0.56 ± 2.93	83.84 ± 1.03	78.91 ± 0.77
50	12.72 ± 0.20	9.20 ± 2.04	84.43 ± 1.09	83.11 ± 0.46
100	25.39 ± 0.41	21.37 ± 2.77	85.62 ± 0.10	84.21 ± 0.50
200	46.12 ± 0.54	37.83 ± 0.81	-	85.31 ± 0.35
400	76.19 ± 0.81	65.67 ± 0.18	-	-
IC_{50} (µg/mL)	**225.87 ± 3.88^{c}**	**287.52 ± 2.92^{b}**	**7.20 ± 0.25^{a}**	**10.03 ± 0.84^{a}**

Values expressed as mean ± SD (n=3). In the last line followed by a different letter (a-c) are significantly different ($p<0.05$). BHA: butylated hydroxyl anisole. IC_{50}: half maximal inhibitory concentration expressed as the necessary concentration to decrease the initial absorbance of DPPH by 50%.

Table 3. Antioxidant capacity of the methanolic extracts of *E. retusa* by the ABTS assay.

Concentration (µg/mL)	**Inhibition** (%)			
	Stems	**Leaves**	**Seeds**	**BHA**
6.25	8.60 ± 3.51	11.83 ± 1.33	65.23 ± 0.48	93.50 ± 0.09
12.5	11.55 ± 0.54	13.20 ± 2.06	91.09 ± 2.16	93.55 ± 0.09
25	24.88 ± 0.77	16.22 ± 1.10	91.80 ± 2.76	93.60 ± 0.16
50	29.21 ± 3.51	27.03 ± 0.66	92.68 ± 0.38	93.60 ± 0.95
100	54.44 ± 0.19	44.33 ± 1.32	93.07 ± 1.29	94.17 ± 0.90
200	70.11 ± 2.08	64.86 ± 0.25	-	-
400	90.05 ± 2.27	84.90 ± 2.86	-	-
IC_{50} (µg/mL)	**91.45 ± 1.33^{b}**	**126.75 ± 3.83^{c}**	**<6.25^{a}**	**<6.25^{a}**

Values expressed as mean ± SD (n=3). In the last line followed by a different letter (a-c) are significantly different ($p<0.05$). BHA: butylated hydroxyl anisole. IC_{50}: half maximal inhibitory concentration declared as the necessary concentration to decrease the initial absorbance of ABTS by 50%.

Table 4. Antioxidant capacity of the methanolic extracts of *E. retusa* by the CUPRAC assay.

Concentrations (µg/mL)	Absorbance			
	Stems	**Leaves**	**Seeds**	**BHA**
6.25	0.09 ± 0.00	0.09 ± 0.00	0.36 ± 0.01	1.90 ± 0.04
12.5	0.11 ± 0.00	0.11 ± 0.02	0.59 ± 0.03	2.48 ± 0.18
25	0.12 ± 0.05	0.14 ± 0.00	0.84 ± 0.14	2.55 ± 0.20
50	0.21 ± 0.00	0.20 ± 0.00	1.29 ± 0.18	2.76 ± 0.11
100	0.33 ± 0.01	0.33 ± 0.01	1.31 ± 0.13	-
200	0.56 ± 0.01	0.54 ± 0.00	1.75 ± 0.24	-
400	0.98 ± 0.01	0.95 ± 0.12	2.72 ± 0.09	-
$A_{0.50}$ (µg/mL)	**173.35 ± 1.21^{c}**	**180.34 ± 0.20^{d}**	**10.64 ± 0.99^{b}**	**<6.25^{a}**

Values expressed as mean ± SD (n=3). In the last line followed by a different letter (a-d) are significantly different ($p<0.05$). BHA: butylated hydroxyl anisole. $A_{0.50}$: A0.5 (mg/mL) corresponding the concentration indicating 0.50 absorbance intensity.

Table 5. Antioxidant capacity of the methanolic extracts of *E. retusa* by the reducing power assay.

Concentration (µg/mL)	Absorbance			
	Stems	**Leaves**	**Seeds**	**Ascorbic acid**
1.5625	-	0.06 ± 0.01	0.19 ± 0.01	0.23 ± 0.01
3.125	0.07 ± 0.01	0.06 ± 0.01	0.20 ± 0.13	0.39 ± 0.01
6.25	0.08 ± 0.01	0.16 ± 0.17	0.36 ± 0.02	0.70 ± 0.08
12.5	0.11 ± 0.00	0.09 ± 0.01	0.53 ± 0.04	0.94 ± 0.05
25	0.15 ± 0.02	0.10 ± 0.06	0.76 ± 0.12	1.19 ± 0.40
50	0.24 ± 0.01	0.20 ± 0.03	1.00 ± 0.06	1.35 ± 0.18
100	0.37 ± 0.03	0.28 ± 0.06	1.13 ± 0.14	1.49 ± 0.03
$A_{0.50}$ (µg/mL)	**>100^{c}**	**>100^{c}**	**11.84 ± 1.72^{b}**	**4.40 ± 0.39^{a}**

Values expressed as mean ± SD (n=3). In the last line followed by a different letter (a-c) are significantly different ($p<0.05$). $A_{0.5}$ (mg/mL) corresponding the concentration indicating 0.50 absorbance intensity.

DISCUSSION

Total bioactive compounds

The phenolic compounds have been intensively investigated due to their antioxidant roles (Shaikh et al., 2015). However, the results of the present study suggest that the phenolic contents were found to be significantly variable between the three organs. These results support the findings indicating that the distribution of secondary metabolites may change depending on plant organ (Karoune et al., 2015). Moreover, seed extracts registered the highest yield. In the same way Joshi et al. (2015) found the highest contents in total phenolic contents in seeds than flowers and leaves of *Cassia auriculata* (Linn.) methanolic extracts.

Likewise to the phenolic contents, *E. retusa* seeds were richer in total flavonoid contents than stems and leaves. Similar results were found by Falleh et al. (2008) hence indicating a notably higher flavonoid contents in *Cynara cardunculus* L. seeds than leaves and flowers.

Several reports have described the use of HPLC-DAD for the characterization of phenolic composition (Ksouri et al., 2009; Falleh et al., 2012; Karoune et al., 2015). For that, in this study was used the HPLC-DAD system to identify phenolic compounds including the major compound as the flavonoid epigallocatechin 3-O-gallate. However, the four phenolic compounds identified are known for the antioxidant capacity (Marinova and Yanishlieva, 2003; Zhang et al., 2012). In comparison to other works on phenolic compounds identified in same genus, luteolin-7-O-glucoside was the predominant flavonoid glycoside in the methanolic extract of aerial parts of *Euphorbia hirta* (Pióro-Jabrucka et al., 2011).

Antioxidant capacity

The chemical complexity of extract and the multifaceted aspects of antioxidant reactivity such as a mechanism of electron/hydrogen donation (Tel et al., 2010) imply to use several methods for demonstrating antioxidant capacity. In fact, in the present study, mainly four methods were used to evaluate the antioxidant capacity of the methanolic extracts of *E. retusa* organs.

Practically, for DPPH test, the lowest IC_{50} value indicated the highest antioxidant activity. IC_{50} values of DPPH and percent inhibition at all concentrations were largely differed between the three organs. This trend is supported by previous studies (Ashraf et al., 2015; Moualek et al., 2016). Seeds showed higher antioxidant capacity without statistical differences ($p>0.05$) respect to the standard BHA and the IC_{50} value of this extract (7.20 µg/mL) was three fold higher as compared to those reported by Falleh et al. (2008) for methanolic extract of seeds of *Cynara cardunculus* L. (IC_{50} = 23 µg/mL). This Result suggests that, the seeds extract contains phytochemical constituents that are capable of donating hydrogen to a free radical to diminish the potential damage.

The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate (Saeed et al., 2012). As shown for DPPH scavenging (Table 2), the result indicates the higher capacity of seed extracts to quench $ABTS^{\bullet+}$ and the half maximal inhibitory concentration of this extract was lower than 6.25 µg/mL as well as the BHA (Table 3). However, stem (91.4 µg/mL) and leave 126.7 µg/mL) extracts were characterized by higher capacity than other data reported by Saeed et al. (2012) for the methanol extract of aerial parts of *Torilis leptophylla* L. (IC_{50} = 179 ± 3 µg/mL).

For the CUPRAC assay the reducing ability of the three organs and the BHA increased with the increase in concentrations between 6.25 and 400 mg/mL. A similar result was reported by Tel et al. (2010) between 50 and 800 µg/mL for *Salvia chionantha* hexane extract for this test. The seed extracts exhibited higher activities at all concentrations and the BHA (Table 4).

In reducing power assay, an increase in absorbance indicates a greater capacity of the extract reducing abilities and stronger antioxidant potential. The methanolic seeds extract was found to be the highest reducer. In the literature reviewed no studies were found on the reducing power of *E. retusa* organs.

As expected, a positive correlation level demonstrated between the phenolic compounds and the antioxidant activities for the four tests. This significant correlation was supported by Oueslati et al. (2012) analyzing *Sueda* species. However, total phenolic and flavonoid contents were higher in seeds followed by stems and leaves. This order has been approved by the antioxidant capacity of all methods tested. These results are corroborated with previous reports on *Tamarix gallica* L. flowers and leaves (Ksouri et al., 2009), concluding that leaves exhibited the lowest phenolic contents and antioxidant activity

CONCLUSIONS

The results revealed that methanolic extracts of stems, leaves and seeds of *E. retusa* showed significant phenolic contents and antioxidant capacity with higher significant values for seed extracts. These results may confirm the interesting potential of *E. retusa* as a valuable natural alternative source to the synthetic antioxidants. More studies should be done to demonstrate the antioxidant activity of the seeds of this species as well as possible phar-

macological activity in diseases that pass through an oxidative stress component.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Lahmadi S	Belhamra M	Karoune S	Kechebar MSA	Bensouici C	Kashi I	Mizab W	Ksouri R
Concepts or ideas			x	x				
Design	x	x	x	x				x
Definition of intellectual content	x		x	x				
Literature search	x							
Experimental studies	x				x	x	x	
Data acquisition	x				x			
Data analysis	x			x	x			
Statistical analysis	x				x	x		
Manuscript preparation	x							
Manuscript editing	x							
Manuscript review	x	x	x	x	x	x	x	x

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Annex 1. Standard compounds used by HPLC-DAD analysis.

No.	Standard Compound	Retention time (min)
1	Ascorbic acid	4.414
2	Gallic acid	8.209
3	Resorcinol	10.308
4	Catechol	12.034
5	Catechin hydrate	14.307
6	Epigallocatechin	14.699
7	Chlorogenic acid	15.752
8	Epicatechin 3-O gallate	16.267
9	Caffeic acid	17.424
10	Syringic acid	17.802
11	p-Coumaric acid	19.692
12	Sinapic acid	19.977
13	Ferulic acid	20.230
14	Myricitrin	20.741
15	Luteolin 7-O glucoside	20.937
16	Trans hydroxycinnamic acid	20.942
17	Coumarin	20.995
18	Isorhamnetin 3-O glucoside	21.100
19	Resveratrol	21.737
20	Rosmarinic acid	21.753
21	Protocatechuic acid ethyl ether	21.773
22	Oleuropein	21.909
23	Isoquercitrin	21.925
24	Ellagic acid	22.278
25	Myricetin	22.457
26	Kaempferol 3-O rutinoside	22.538
27	Isorhamnetin 3-O rutinoside	22.833
28	Quercetin	24.171
29	Naringenin	24.320
30	Trans cinnamic acid	24.360
31	Laricitrin	24.749
32	Luteolin	24.787
33	Kaempferol	25.455
34	Isorhamnetin	25.762
35	Amentoflavone	26.873
36	Apigenin	25.897
37	Cirsimaritin	26.948