Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Original article doi: 10.12980/jclm.4.2016J6-118

Total phenolic compounds, antioxidant potential and α-glucosidase inhibition by Tunisian *Euphorbia paralias* L.

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ARTICLE INFO

Article history:
Received 11 Jul 2016
Received in revised form 20 Jul, 2nd revised form 26 Jul, 3rd revised form 31 Jul 2016
Accepted 2 Aug 2016
Available online 11 Aug 2016

Keywords:
Euphorbia paralias L.
Polyphenolic compounds
Antioxidant activity
Anti-α-glucosidase activity

ABSTRACT

Objective: To examine the potential antioxidant and anti-α-glucosidase inhibitory activities of Tunisian *Euphorbia paralias* L. leaves and stems extracts and their composition of total polyphenol and flavonoids.

Methods: The different samples were tested for their antiradical activities by using 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays. In α-glucosidase activity, α-glucosidase (0.3 IU/mL) and substrate, 2 500 μmol/L *p*-nitrophenyl α-D-glucopyranoside were used; absorbance was registered at 405 nm.

Results: The leaves acetonic extract exhibited the strongest α-glucosidase inhibition [IC_{50} = (0.0035 ± 0.001) μg/mL], which was 20-fold more active than the standard product (acarbose) [IC_{50} = (0.07 ± 0.01) μg/mL]. Acetonic extract of the leaves exhibited the highest quantity of total phenolic [(95.54 ± 0.04) μg gallic acid equivalent/mg] and flavonoid [(55.16 ± 0.25) μg quercetin equivalent/mg]. The obtained findings presented also that this extract was detected with best antioxidant capacity [IC_{50} = (0.015 ± 0.01) μg/mL] against DPPH and a value of IC_{50} equal to (0.02 ± 0.01) μg/mL against ABTS. Positive relationship between polyphenolic content of the tested *Euphorbia paralias* L. leaves and stems extracts and its antioxidant activity (DPPH and ABTS) was detected. Elevated positive linear correlation was got between ABTS and total phenolic (R^2 = 0.751).

Conclusions: The findings clearly demonstrate that the use of a polar solvent enables extraction of significant quantities of phenol compounds and flavonoids.

1. Introduction

Diabetes mellitus (DM) is an international metabolic disease described by elevated blood glucose levels. In 2013, 382 million of population in the world is living with DM, 90% of them were affected by type 2 diabetes, and the number is predicted to raise to 592 million by 2035 with the growth of urbanization, improvement of living standard and modifications of humans' diet practice[1,2]. The usable synthetic medicines for the curing of DM mainly are pricey and engender dangerous side impacts[3,4]. The detection of medicinal plants for new therapeutic products is, thus, a considerable object for researchers. Mostly, the plant based medicines are biodegradable, safe, and inexpensive, having less side effects[5]. Many studies also suggested the application of medicinal plant extracts to antidiabetic treatments due to fewer side effects than those of synthetic drogues[6-10]. Another benefit is that natural compounds may be safely consumed in the quotidian regime, thereby decreasing the risk of DM[11]. As the

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Foundation Project: Supported by the Ministry of High Education and Scientific Research, MHSSR of Tunisia (Grant No. 11/TM06).
The journal implements double-blind peer review practiced by specially invited international editorial board members.

pathogenesis of DM implies oxidative stress, antiradical treatment must have an interest value in its therapies. Several processes in animal examples of diabetes and diabetic sick people have tried to establish the docket of antioxidants on the treatment or prevention of diabetes aggravation[12]. Therefore, the search for antidiabetic and antioxidant drug components from plants has been appealing increasing attentiveness in latest years. *Euphorbia paralias* L. (*E. paralias*) is also known as *Tithymalus paralias* L.[13]. The species of *Euphorbia*, mostly have antileukaemic property applied extensively in popular medicine for healing different types of cancer like liver, stomach and uterus, used, also to treat sickness with inflammation, as a purgatory. The use of *Euphorbia* species in the remedy of skin illness and asthma has been postponed[14-16]. Previous phytochemical studies on *E. paralias* led to the identification of several terpenes such as pre-segetanin, segetanin A, segetanin B[17], segetenes A, segetenes B, paralinone A, paralinone B[18], segetane diterpenes, jatrophanes and paralianes[19-23]. Some flavonoids were isolated from this plant, such as quercetin, hyperoside and kaempferol 3-β-D-glucoside[24]; one phytosterol was isolated, β-sitosterol[24].

The goal of the present work is the dosage of flavonoids and polyphenols in the leaves and stems of *E. paralias* extracts. Further in this study, the assay of α-glucosidase inhibitory activity and the inhibition type were conducted *in vitro*. The antioxidant activities of these extracts were also tested using different assays. These assays may be used for preliminary observations in the evaluation of pharmacological activities and also to check the therapeutic effects of this plant.

2. Materials and methods

2.1. Chemicals and reagents

The solvents employed in the experiments (chloroform and acetone) were acquired from Merck (Darmstadt, Germany). Potassium persulphate, 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), 4-p-nitrophenyl-α-D-glucopyranoside (4-pNPG) and α-glucosidase (isolated from *Aspergillus niger*) were retained from Sigma-Aldrich.

2.2. Plant materials

The plant *E. paralias* was collected in the month of February in 2010 in the region of Monastir. This species was kindly determined by Pr. Fethia Harzallah Skhiri. A voucher specimen (*E. paralias*) was filed in the herbarium of the Laboratory of Transmissible Diseases and Biological Active Substances, Faculty of Pharmacy.

2.3. Preparation of extracts

The leaves and the stems were dried for two weeks at ambient temperature and reduced to raw powder. The powdered specimen (250 g) was separately extracted by maceration with chloroform and acetone. A repetitive extraction of the same material three times with solvent was carried out. The samples obtained were concentrated to dryness with a rotary evaporator under reduced pressure after filtration. Four extracts were obtained and maintained at 4 ˚C before analysis.

2.4. Chemical analysis

2.4.1. Total phenolics

The quantity of total phenolics in the four extracts prepared was detected following the protocol of Velioglu *et al.*[25] which uses Folin-Ciocalteu reagent. A volume of 100 µL of the extract (1 mg/mL) was moved into a hemolysis tube and 750 µL of Folin-Ciocalteu reagent was appended and blended. After incubation period (5 min at 25 ˚C), we added 0.75 mL of Na_2CO_3 (saturated sodium carbonate solution) to the concoction and then it was slowly mixed. After 90 min at 25 ˚C, the optical density was read at 725 nm using an UV-vis spectrophotometer. The total phenolic content of *E. paralias* extracts was manifested as µg gallic acid equivalents per milligram of dry weight (µg GAE/mg DW) by the calibration curve with gallic acid. We used a calibration curve which ranged from 0 to 250 µg/mL ($R^2 = 0.99$).

2.4.2. Total flavonoids

The Lamaison and Carnet procedure[26] was worn to establish the total flavonoid content of the *E. paralias* samples. Nearly 1 500 µL of samples were supplemented to equal volumes of a solution of 2% $AlCl_3 \cdot 6H_2O$. The mixture was loudly agitated. After incubation (10 min), the optical density was recorded at 367 nm. The total flavonoids tenor was manifested as µg quercetin/mg dry weight (mg QE/g DW), by the calibration curve of quercetin. We used a calibration curve which ranged from 0 to 50 µg/mL ($R^2 = 0.99$).

2.5. Antiradical activity

2.5.1. DPPH test

This assay seek the ability of the samples to trap the steady radical (DPPH) created in solution by yielding a hydrogen atom or an electron[27]. If the samples have the capability to snare the DPPH free radical, the first blue/purple solution will transform to a yellow color due to the formation of diphenylpicrylhydrazyl. Later 500 µL of each extract concentration was blended employing the equal volume of $DPPH^{\bullet}$ ethanolic solution. The optical density was read at 517 nm wavelength, after the incubation at 25 ˚C during 30 min. A blend of 500 µL of $DPPH^{\bullet}$ solution and 500 µL of ethanol was considered as a blank[28]. Reduction in optical density induced by the tested extracts was compared to the positive control BHT. IC_{50} values calculated signify the concentration needful to scavenge 50% of $DPPH^{\bullet}$ radicals. Findings were manifested in inhibition percentage at various extract concentrations (µg/mL). Inhibition of free radical DPPH in percentage was calculated like that:

$$\text{Inhibition } (\%) = (A_{control} - A_{extract})/A_{control} \times 100$$

where $A_{control}$ is the absorption of the control reaction (holding all reagent unless the test sample), and $A_{extract}$ is the absorption of the extract tested.

2.5.2. ABTS test

Antioxidant activity was realized by using the $ABTS^{\cdot+}$ free radical decolorization test provided by Re *et al.*[29] with a few modifications. Shortly, the preformed radical monocation of ABTS was produced by reacting 2.45 mmol/L $K_2S_2O_8$ with ABTS solution (7 mmol/L). After 15 h of rest in the obscure at ambient temperature, the solution was diluted with ethanol to obtain the absorption of (0.7 ± 0.2) units at 734 nm. Extracts were separately dissolved in ethanol to yield the next concentrations (from 0.015 mg/mL to 1 mg/mL). After that 0.01 mL of every concentration was added to 0.99 mL of diluted $ABTS^{\cdot+}$. After incubation during 20 min, the optical density was read. The inhibition percentage of $ABTS^{\cdot+}$ was obtained by the next formula:

Inhibition (%) = $((A_{control} - A_{extract})/A_{control}) \times 100$

where $A_{control}$ is the absorption of the control reaction (holding all reagent unless the test sample), and $A_{extract}$ is the absorption of the extract tested.

2.6. α-Glucosidase inhibition assay

The method used in this activity was that of Tao *et al.*[30] with a few modifications. The α-glucosidase reaction mixture retained 250 μL of sample (different concentrations) in dimethylsulfoxide, 0.3 IU/mL α-glucosidase in phosphate buffer (pH 6.9) and 2.5 mmol/L (4-pNPG). The incubation was carried out during 15 min in a water bath at 37 °C. Control tubes regrouped just enzyme, substrate and dimethylsulfoxide. The positive controls contained acarbose instead of the species samples. The optical density of the resulting p-nitrophenol was detected at 405 nm and was examined straight to the activity of the enzyme. Every extract was carried out in triplicate. The inhibition percentage by acarbose and samples were studied using the next equation:

Inhibition (%) = (1 – ΔDO extract/ΔDO control) × 100

where ΔDO extract = DO extract $_{(t = 15\ min)}$ – DO extract $_{(t = 0\ min)}$, ΔDO control = DO control $_{(t = 15\ min)}$ – DO control $_{(t = 0\ min)}$

The IC_{50}, which means the concentration of the extract intended to inhibit 50% of the enzyme was examined for every extract.

2.7. Kinetics study of α-glucosidase

Lineweaver-Burk plot test was used to establish the method of inhibition of extracts. The kinetics was determined by using different concentrations of 4-p-nitrophenyl-α-D-glucopyranoside as a substrate in the attendance of different concentrations of samples. *Ki* show the equilibrium constant for the binding of extracts to α-glucosidase. The inhibition constant *Ki* value was determined from the secondary plots constructed using slopes or y-intercepts of Lineweaver-Burk plots. The initial rates of reaction were detected using calibration curves made using different concentrations of the substrate.

2.8. Statistical analysis

The findings were determined as the pass mark ± SE for minimum three experiments for every extract. The IC_{50} (ABTS, α-glucosidase and DPPH) values were measured by linear regression analysis. The data were exposed to ANOVA, and Duncan's multiple range test was worn to compare averages. Statistical analysis was executed with the SPSS statistical software program (SPSS v.16). P values < 0.05 were considered as significant.

3. Results

3.1. Total phenolics

According to Table 1 below, the polyphenol contents recorded in gallic acid equivalent in μg per mg of sample showed that the acetone samples of the leaves and the stems were rich in phenolic compounds [(95.54 ± 0.04) μg GAE/mg] and [(29.66 ± 0.08) μg GAE/mg], followed by chloroform extract of the leaves [(11.03 ± 0.03) μg GAE/mg] and the chloroform extract of the stems [(9.75 ± 0.01) μg GAE/mg].

3.2. Total flavonoids

The highest flavonoid content was found also in the leaves acetonic extract [(55.16 ± 0.25) μg QE/mg] followed by the stems acetonic extract and the stems chloroformic extract [(26.25 ± 0.41) μg QE/mg and (20.87 ± 0.01) μg QE/mg, respectively]. The lowest value was shown with the leaves chloroformic extract [(1.87 ± 0.18) μg QE/mg].

3.3. Antioxidant activity

3.3.1. DPPH test

The findings from the radical scavenger tests for all *E. paralias* samples are cited in Table 1 as IC_{50} (μg/mL); IC_{50} signify the concentration of the sample capable to trap moiety of the DPPH free radical existing in the test solution. The greatest results were got with the leaves acetonic extract [IC_{50} = (0.015 ± 0.01) μg/mL], the stems acetonic extract [IC_{50} = (0.05 ± 0.01) μg/mL], the leaves chloroformic extract [IC_{50} = (0.1 ± 0.01) μg/mL] and the stems chloroformic extract [IC_{50} = (0.15 ± 0.09) μg/mL], respectively. The BHT was used as standard in the *in vitro* antioxidant activity.

3.3.2. ABTS test

$ABTS^{\cdot+}$ test is founded on the anti-radical capacity to respond with ABTS radical cation created in the test system. $ABTS^{\cdot+}$ is a process founded on reduction of the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) radical. $ABTS^{\cdot+}$ have been extensively used to determine the antioxidant ability of natural sample based on their capability to decrease the radical cation, reactions of $ABTS^{\cdot+}$ with free radical scavengers found in the plant sample take place quickly and may be evaluated by next reduction in the extract optical density at 734 nm[31]. The effect of *E. paralias* extracts on ABTS radical cation scavenging property is shown in Table 1. The four extracts presented interesting ABTS radical scavenging property. The leaves acetonic sample executed the best antioxidant activity [IC_{50} = (0.02 ± 0.01) μg/mL] followed by the stems acetonic extract [IC_{50} = (0.04 ± 0.02) μg/mL].

Table 1
Anti-radical property and α-glucosidase inhibition of *E. paralias* extracts.

Extracts	Total phenolic content (μg GAE/mg)	Total flavonoid content (μg QE/mg)	IC_{50} (mg/mL)		
			DPPH· scavenging ability	ABTS· scavenging ability	α-Glucosidase inhibition
Stems acetonic extract	29.66 ± 0.08	26.25 ± 0.41	0.05 ± 0.01^{ab}	0.04 ± 0.02^{b}	0.006 ± 0.001^{b}
Leaves acetonic extract	95.54 ± 0.04	55.16 ± 0.25	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.0035 ± 0.001^{a}
Stems chloroformic extract	9.75 ± 0.01	20.87 ± 0.01	0.15 ± 0.09^{c}	0.10 ± 0.02^{d}	0.035 ± 0.01^{c}
Leaves chloroformic extract	11.03 ± 0.03	1.87 ± 0.18	0.10 ± 0.03^{bc}	0.09 ± 0.01^{c}	0.015 ± 0.009^{a}
BHT	-	-	0.02 ± 0.01^{a}	0.05 ± 0.01^{b}	-
Acarbose	-	-	-	-	0.07 ± 0.01^{d}

Different letters in the same column show significant differences ($P < 0.05$). IC_{50} values are shown as the average ± SD of three independent experiments.

3.3.3. Relationship between anti-oxidant activity and flavonoid, phenolic contents

One of the objectives of this paper was to consider the correlation between antioxidant property and phenolic compounds of *E. paralias* samples. Correlation between the results of different antioxidant tests is shown in Figures 1 and 2. A positive linear correlation between the values for the antioxidant activity (DPPH and ABTS) and total phenolic content was observed (R^2 = 0.568 and R^2 = 0.751). A good correlation was equally reported for total flavonoid content (R^2 = 0.676) for the ABTS test. The lowest correlation coefficients were between DPPH values and total flavonoid (R^2 = 0.495).

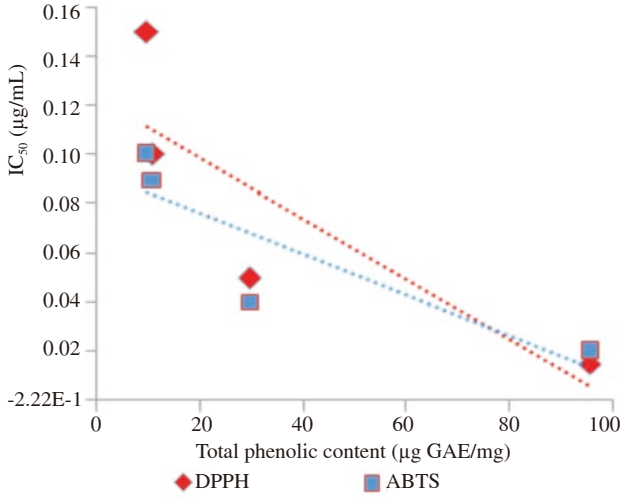

Figure 1. Relationship between antioxidant activities and total phenolic content.

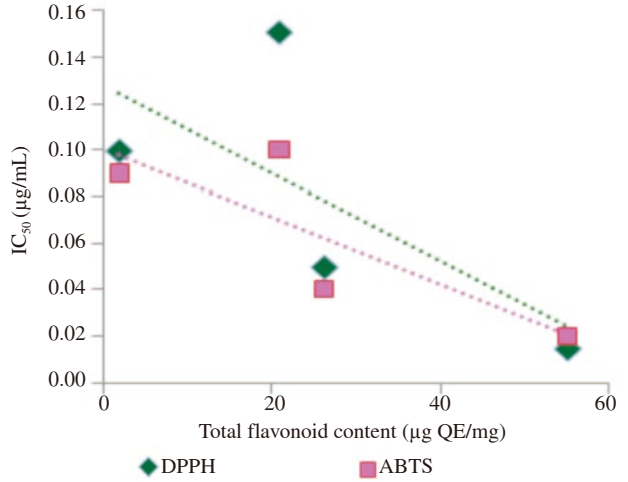

Figure 2. Relationship between antioxidant activities and flavonoid content.

3.4. α-Glucosidase inhibition assay

The IC_{50} values for the α-glucosidase inhibitory activities of *E. paralias* samples and acarbose as the standard are summarized in Table 1. As shown in this table, all extracts exhibited an important α-glucosidase inhibition. The leaves acetonic extract [IC_{50} value of (0.0035 ± 0.001) μg/mL] possessed the highest inhibition followed by the stems acetonic extract [IC_{50} value of (0.006 ± 0.001) μg/mL]. The stems chloroformic sample and the leaves chloroformic sample also exhibited considerable α-glucosidase inhibition [IC_{50} = (0.0035 ± 0.01) μg/mL and (0.015 ± 0.009) μg/mL, respectively]. This biological activity of all *E. paralias* samples was higher than that of acarbose [(0.07 ± 0.01) μg/mL]. The α-glucosidase inhibitory property of *E. paralias* samples was analyzed here, for the first time.

3.5. Kinetics of α-glucosidase activity

The kinetic studies were performed using Lineweaver-Burk plot analysis. For this purpose, the leaves acetonic extract which presented the lower values of IC_{50} = (0.0035 ± 0.001) μg/mL was shosen to give the mode of inhibition (uncompetitive, non-competitive or competitive) of α-glucosidase. In the enzyme kinetic studies, the rate of the enzyme activity was studied at three various concentrations of leaves acetonic extract using five different concentrations of 4-pNPG (10 000 μmol/L, 5 000 μmol/L, 2 500 μmol/L, 1 250 μmol/L and 625 μmol/L). The Lineweaver-Burk plots exhibited that this extract inhibited α-glucosidase in a competitive manner (Figure 3). In effect, the plots intersected the Y-axis.

Thus, the addition of the leaves acetonic extract in the reaction medium resulted in a modification in the Michaelis-Menten constant (K_M) while keeping the same value of maximum velocity (*Vmax*) of α-glucosidase (*Vmax* = 0.14 ΔDO/min). The values of the kinetic parameters of α-glucosidase in the absence and presence of the leaves acetonic sample are summarized in Table 2.

Table 2
The kinetic parameters of α-glucosidase in the presence and absence of the *E. paralias* leaves acetonic extract.

Inhibitors (I)	Inhibition mode	*Vmax* (ΔDO/min)	K_M (mmol/L)	K_{Mapp} (mmol/L)
I = 0	-	0.14	5.88	-
I = 150 μg/mL	Competitive inhibition	0.14	-	6.66
I = 300 μg/mL	Competitive inhibition	0.14	-	9.09
I = 600 μg/mL	Competitive inhibition	0.14	-	16.66

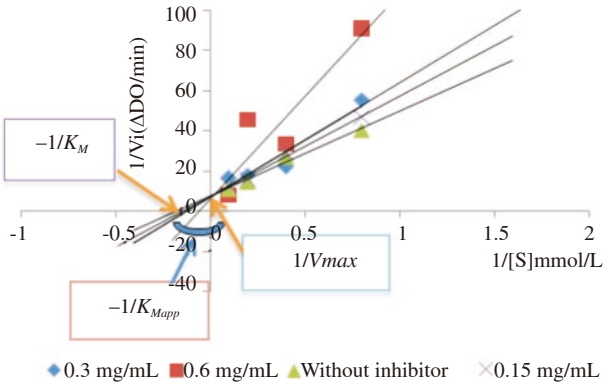

Figure 3. Double-reciprocal plot of the initial velocity (*Vmax*) of the hydrolysis reactions catalyzed by α-glucosidase at different substrate concentrations [S] in the presence and absence of the leaves acetonic sample.

The findings indicate the average of three independent triplicate experiments.

The competitive mode illustrated that this extract bound to the active site of the enzyme, competing with the substrate, to delay the substrate conversion.

To establish the inhibition constant (*Ki*) of the most active sample, the secondary replots of Lineweaver-Burk plots were plotted (Figure 4). The *Ki* value derived from secondary plots was 0.12 mg/mL (Figure 4) and the point of intersection of lines described the value of *Ki*, which was derived by using slope (obtained from Lineweaver-Burk plot) versus inhibitor concentrations values (150 µg/mL, 300 µg/mL and 600 µg/mL).

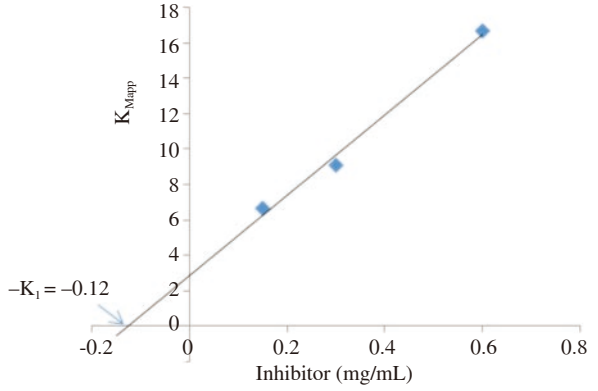

Figure 4. Secondary plots of slopes against leaves acetonic extract concentrations to calculate *Ki*.

4. Discussion

The medicinal plants have quite a long time story in the healing of several illnesses including DM. The majority of these medicinal plants which possess anti-diabetic activity have antioxidant activity[32]. Many researchers have demonstrated that in stressful conditions free radicals are produced in excess, causing oxidative stress. Oxidative stress takes place when there is unsteadiness between free radical formation and antioxidant defense capability[33]. This oxidative stress generally produces or aggravates chronic difficult curable diseases like diabetes[34]. In this paper, the total phenolic compounds, antioxidant potential and the α-glucosidase inhibition by extracts of *E. paralias* were studied for the first time.

Comparing our results with the literature, we find similar results; Sheliya *et al.*[35] worked on *Euphorbia hirta* extracts and showed that the methanolic extract presented total flavonoids content in the range of (55.35 ± 2.02) mg QE/g. This value is very close to that found for the *E. paralias* leaves acetonic extract [(55.16 ± 0.25) mg QE/g]. Another study has shown that the total polyphenols content of *Euphorbia rayleana* whole plant varies from (30.97 ± 0.46) mg GAE/g to (63.68 ± 0.43) mg GAE/g, while the dosage of flavonoids in the plant varies from to (18.89 ± 0.41) mg QE/g to (47.47 ± 0.71) mg QE/g[36].

Concerning the biological activity, one study was found testing the antioxidant activity of *E. paralias* samples, using the DPPH method; thus, the Egyptian *E. paralias* ethanolic extract showed an interesting antioxidant activity (81.1%) and the water extracts of this plant presented a moderate antioxidant activity (51.8%)[37]. While in the $ABTS^{\cdot+}$ assay, it wasn't tested before, and confirmed the encouraging antioxidant property of the leaves acetonic extract [(20.00 ± 0.01) µg/mL]. The *E. paralias* acetone extracts had an interesting ability to quench DPPH and ABTS radicals due to the presence of phenolic products with hydroxyl group tied to the aromatic ring structures[38]. It was considered that these acetone extracts were a natural origin of strong natural antioxidant property. Its great total polyphenols and total flavonoids contents values denoted that the machinery of anti-radical action of these samples was hydrogen donor and they would finish the oxidation process by changing free radicals to the steady shape. The polyphenols in extracts are probably responsible for the high anti-radical property of *E. paralias* samples. However, this activity is not limited to phenolics content but also in the presence of other antioxidant secondary metabolites. These different correlation coefficients support the necessity to use multitude of methods to test antioxidant activity of plants. For the α-glucosidase inhibitory activity of *E. paralias* extracts, few studies have tested the anti-α-glucosidase activity of the species belonging to the genus *Euphorbia*. Thus, three extracts of *Euphorbia hirta* whole plant (aqueous, hydroalcoholic, and methanolic) showed values of IC_{50} of the order of 0.213 mg/mL, 0.146 mg/mL and 0.078 mg/mL, respectively[35]. These values are lower than those found by our extracts, ranged from 3.5 µg/mL to 35 µg/mL. The inhibition type of the most active extract (leaves acetonic extract) was determined and it is a competitive inhibition with value of *Ki* (120 µg/mL). Sheliya *et al.*[35] investigated the kinetics of *Euphorbia hirta* methanolic extract showing the more interesting value of IC_{50} = 0.078 mg/mL; this extract showed a mixed non-competitive inhibition.

This work assumes to discuss the potential of extract of *E. paralias* (leaves and stems) of Tunisia as sources of natural α-glucosidase inhibitors and antioxidants. The richness of leaves in bioactive compounds (polyphenolic and flavonoids) was noted.

Two anti-radical tests were successfully conducted so as to test the anti-radical property of the plant samples, with similar results being obtained. The leaves and the stems acetonic extracts demonstrated potent antioxidant properties and α-glucosidase inhibition, while the leaves and the stems chloroformic samples presented moderate inhibitory property versus ABTS and DPPH radicals. The presence of phenolic compounds in our samples seems to be the good reason for the antioxidant activities and α-glucosidase inhibition.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This research was supported by the Ministry of High Education and Scientific Research, MHSSR of Tunisia (Grant No. 11/TM06).

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