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Antioxidant and Free Radical-Scavenging Activity of *Tulipa Systola* Roots, Leaves and Flowers Collected in the Kurdistan Region of Iraq

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Abstract
We report the first investigation on the antioxidant and antiradical properties of extracts of roots, leaves, and flowers of *Tulipa systola* Stapf, which is used as a pain-relief in the traditional medicine of the Kurdistan region of Iraq. The study has revealed that all parts of the plant contain significant amounts of phenolics and other antioxidant metabolites; In the Folin-Ciocalteu assay the total antioxidant capacity of the ethanol extract of flowers was even higher than that of ascorbic acid. The DPPH radical scavenging activity, the hydrogen peroxide and Fe (III) reducing powers have also been measured. The values were different for the different extracts, clearly reflecting the great variety of contents of secondary metabolites. These compounds possibly have different redox properties and radical quenching properties in vitro, and exert their effects through different mechanisms. Results are in great subtend to become a starting point for in vivo investigation in the next steps, isolation and characterization of the responsible antiradical and antioxidant secondary metabolites.
Keywords: *Tulipa systola*, Polyphenols, Flavonoids, Antioxidant and antiradical scavenging activities.

1. Introduction

The genus *Tulipa* (Liliaceae) is of great economic, horticultural, aesthetical, ecological, conservational, and taxonomic interest. It has attracted a great deal of attention from the Dutch Tulipomania of February 1637 until the today export and tourism boom, which undoubtedly makes the tulip the unofficial national flower of The Netherlands. Wild tulips occur in temperate regions, ranging from the southern Balkans to Siberia and west China, North Africa (Algeria), the Mediterranean East, and the Near East (Iran, Israel, Jordan, Turkey, etc.). [(1)]
The centre of diversity of the genus is in the Pamir and Hindu Kush mountains, and the steppes of Kazakhstan. Some species have established themselves elsewhere: for example, *T. sylvestris* L. grows on old estates in Britain, The Netherlands, the United States, and Sweden, while the so-called Neotulipae, e.g. *T. marjoletii*, *T. didieri*, or *T. rubidusa*, grow in Western Europe.[(2)]

Peoples living on the mountains of the Kurdistan region in Northern Iraq make a large use of herbs in the local traditional medicine. Among them, *T. systola*, which grows under and between rocks, is very popular as an herbal anti-inflammatory remedy and pain-relief; two or three fresh bulbs eaten by the patient particularly during inflammation and birth pain. Therefore, we considered it to be interesting to evaluate the antiradical and antioxidant activity of *T. systola* and to correlate them with the contents of flavonoids and other metabolites in the extracts of different parts of the plant.

Actually, excess oxidants and free radicals in cells are considered to be involved in the origin and progression of many pathological conditions, including aging diseases, neurological disorders, inflammation, and even cancer. In particular, excess reactive oxygen species (ROS), such as singlet oxygen, superoxide, peroxyl and hydroxyl radicals, give rise to the so-called oxidative stress, resulting in severe cell damages.

It is, therefore, believed that the risk of chronic diseases can be reduced and disease progression can be prevented by enhancing the body's natural antioxidant defences or by supplementing them with effective dietary antioxidants which commonly used in foods, have side-effects. [(3, 25-29)]

This assumption is based on the fact that phenolic plant extracts have been shown to neutralize free radicals in various model systems. [(4)] However, the effectiveness of an antioxidant in vivo depends on several factors, which are impossible to reproduce in vitro; among them, the type of free radicals and oxidants, their mechanism of formation and place of origin, and the presence of the damage target are particularly important.[(5)]

Well aware of these limitations, we thought however, that the traditional uses of *T. systola* might receive some scientific evidences from the discovery of high antioxidant and antiradical activities in extracts.

2. Results and Discussion

To examine the antioxidant and antiradical activities of the different parts of *T. systola*, and to direct further investigations towards the most active extracts, flowers, leaves, and roots were separately defatted with petroleum ether, and then extracted with EtOH, followed by 70% aqueous EtOH. Subsequently, each extract was submitted to selected antioxidant and antiradical activity assays in vitro.

2.1. Total Antioxidant Capacity and Radical Scavenging Capability

Although the reducing capacity of a sample is strictly not directly related to its radical scavenging capability, the latter is a very important parameter of antioxidants and it is often considered a measure of the antioxidant capacity. In fact, one important function of many antioxidants is to suppress free radical-mediated oxidation by scavenging radicals through one-electron transfer or $H^{\cdot}$ donation to free radicals such as $^{\cdot}OH$, $O2^{\cdot-}$, $R^{\cdot}$, $RO^{\cdot}$, and $ROO^{\cdot}$, converting them to an even electron species and generating a more stable, less reactive radical. Such substances have been defined primary or radical chain-breaking antioxidants.[6] In this context, antioxidant capacity assays may then be broadly classified as one-electron-transfer (ET) and hydrogen atom transfer (HAT)-based assays, though in some cases, these two mechanisms may not be differentiated with distinct boundaries.
In this investigation the radical scavenging capability of the different extracts of *T. systola* were determined with four assays: the Folin-Ciocalteu assay, the ammonium molybdate reducing power, the DPPH radical scavenging activity, and the Fe (III) reducing power.

2.2. Folin–Ciocalteu assay and ammonium molybdate reducing power

ET-based assays differ from each other for the use of different chromogenic redox reagents with different standard potentials. At first, the radical scavenging activity of each extract of *T. systola* was determined with the well-known *Folin–Ciocalteu reagent* (FCR),[7] where the oxidizing reagent is a molybdophosphotungstic heteropolyacid comprised of $3H_2O{\cdot}P_2O_5{\cdot}13WO_3{\cdot}5\ MoO_3{\cdot}10H_2O$, in which the hypothesized active center is Mo(VI). A second test, based on the use of a simplified chromogenic redox reagent, namely the sole *ammonium molybdate*, [8, 9] was also performed for comparison. Indeed, the two methods give essentially the same kind of information, though the first assay expresses the reducing activity as % gallic acid equivalence, whereas in the second one IC_{50} values are compared with that of ascorbic acid.

The results of the Folin-Ciocalteu test are displayed in Fig. 1. The ethanol extract of *T. systola* flowers (TF1) contained the highest content (52.662%) of reducing substances among the different extracts, while the contents of the other ones were significantly lower and not very different from each other.

In the second test with the exception of the TR2 extract, all the other extracts exhibited from moderate to high antioxidant power

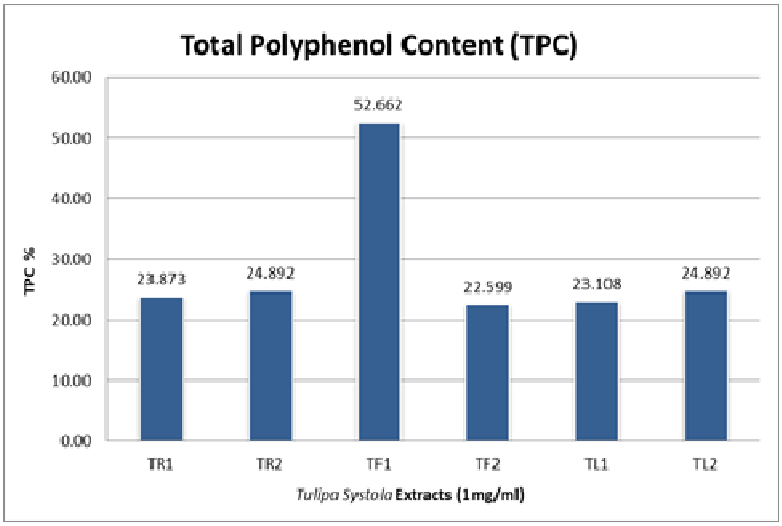

Figure 1. Total polyphenol content for *T. systola* ethanol and 70% aqueous ethanol extracts expressed as % gallic acid equivalent (mg/g).

In fact, the IC_{50} values, compared to the reference ascorbic acid were, in the order: TF1, IC_{50} 0.594 ± 0.001 > ascorbic acid, IC_{50} 0.869 ± 0.001 > TL1, IC_{50} 2.37 ± 0.001 > TF2, IC_{50} 2.71 ± 0.001 > TR1, IC_{50} 2.96 ± 0.001 > TL2, IC_{50} 4.028 ± 0.003. Thus TF1 showed a total antioxidant activity even higher than ascorbic acid. The results of the two tests were comparable, except for TL2, the activity of which resulted to be low in the second assay.

2.3. DPPH radical scavenging activity

A simple to perform, popular test to measure the radical scavenging activity of a sample is by measuring the fluorescence decay of the stable radical DPPH [2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl], resulting from one-electron transfer from the sample.[10] Thus the extent of decolorization, namely the decrease in absorbance, is proportional to the number of electrons gained by the DPPH molecules.

Compared to the reference ascorbic acid the IC_{50} values of the most active extracts were, in the order:

TF1, IC_{50} 103.84 ± 0.042 µg/ml > ascorbic acid, IC_{50} 112.74 ± 0.042µg/ml > TF2, IC_{50} 154.21 ± 0.042 µg/ml > TL2, IC_{50} 213.26 ± 0.042µg/ml. The other parts of the plant showed low radical scavenging properties.
The TF1 extract resulted, again, significantly more active than the reference ascorbic acid and the other extract.

2.4. Fe (III)-reducing power

Fe (III) reduction is often used as an indicator of the electron donating activity. In this assay, the presence of antioxidants in a sample would result in the reduction of Fe (III) to Fe (II) by one electron donation. The amount of Fe (II) formed can be monitored by measuring the formation of the Perl's Prussian Blue complex KFe[Fe(CN)6] at 700 nm. An increasing absorbance at 700 nm would thus indicate an increasing reducing ability.

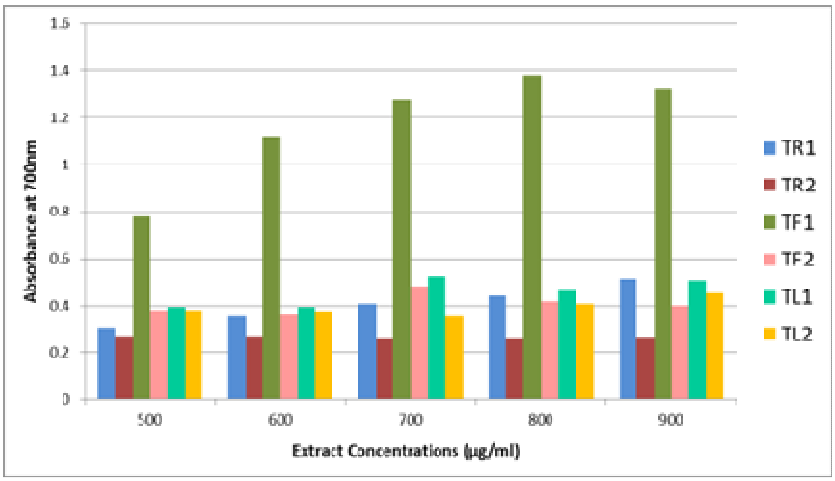

Figure 2. Bar chart for the Fe (III) reducing power of *T. systola* extracts .

All extracts showed a Fe (III) reducing activity, from low to moderate, except for the TF1 extract that exhibited a remarkable, dose-dependent, reducing power, reaching the highest value at 800µg/mL. This activity might be related to the considerably high phenolic content of TF1 (figure 3), and the reducing capacity of this extract may serve as a significant indicator of its potential antioxidant activity and thus its capacity to withstand the free radical stress [(11)].

2.5. Reduction of hydrogen peroxide

One important function of antioxidants is to suppress free radical-mediated oxidation by inhibiting the formation of free radicals, for example ·OH, by reducing hydroperoxides and hydrogen peroxide and by sequestering metal ions through complexation/chelation reactions (secondary or preventive antioxidants). Thus, the capacity of an antioxidant to remove H_2O_2 is very important. [(12, 13)]

The hydrogen peroxide reducing assay was carried out following the procedure of Ruch and co-workers.[(14)] The principle of this method is based on the decrease of the H_2O_2 absorbance at 230 nm upon oxidation.

The extracts TR1 and TL1 of *T. systola* were capable of scavenging hydrogen peroxide in a concentration-dependent manner. The IC_{50} values were 366.88 ± 0.0035µg/ml for TR1 and 383.55 ± 0.235 µg/ml for TL1, respectively, compared to 264.04 ± 0.008 µg/ml for ascorbic acid.

2.6. Total Phenolic Content

Among plant antioxidant and antiradical substances in vitro, simple phenols and polyphenols are the most common components of raw extracts. Besides a significant antioxidant activity, antitumoral, antiviral and antibiotic properties are frequently reported for plant phenols, which have often been identified as active principles of numerous folk herbal medicines. It was, therefore, interesting to measure the total phenolic content in the different extracts of *T. systola* to estimate their contribution to the beneficial effects of the plant, in particular to the total antioxidant activity. The method is based on the spectrophotometric determination of the azo-chromophore absorbance at 415 nm and the phenolic contents are expressed as mg quercetin equivalent/g of dry sample (Fig. 3).

Interestingly, both TR1 and TR2 extracts exhibited higher phenolic contents compared to the extracts of flowers and leaves. This result was, indeed, rather unexpected, since TR1 and TR2 were, in general, among the least active extracts in the antioxidant assays. Other antioxidant substances, in addition to phenolics, must therefore contribute to the high antioxidant capacity of the most active extracts, such as TF1.

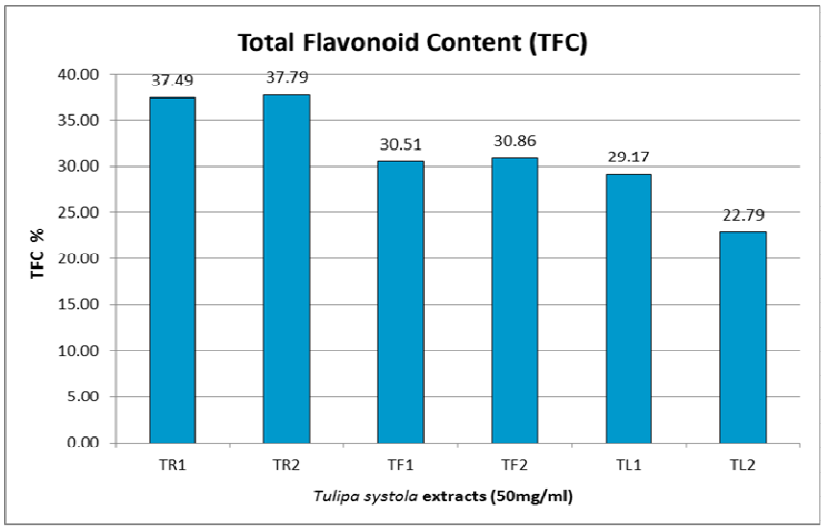

Figure 3. Total flavonoid content of *T. systola* ethanol and 70% aqueous ethanol extracts expressed as quercetin equivalent (mg/g).

3. Experimental

3.1. Material and instruments

Gallic acid, quercetin, L-ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl DPPH (Sigma Aldrich), Folin-Ciocalteau reagent, sodium nitrite, sodium hydroxide, sodium sulphate, sodium carbonate, hydrogen peroxide, potassium ferricyanide, trichloroacetic acid (BDH chemicals Ltd. poole England), ammonium molybdate (Fisher scientific Ltd. UK), methanol, ethanol, petroleum ether (Scharlab S.L Spain), sulphuric acid (Karaj co. Iran); spectrophotometer 7305, series no.2048 Tenway, UK; ultra-sonic cleaner bath, capacity 15L, gp-060/s.; rotary evaporator R-1001, GW; grinding mill, Beijing co., Ltd; electrical balance, Sartorius type 1801 (110-0.1mg); micropipette, transferpette® 04Z7139, Germany.

3.2. Plant material

Tulipa systola Stapf. was collected in April 2014 on the Korek Mountains in Rewanduz - Erbil / Kurdistan region. The materials were identified and classified from Education Salahaddin University Herbarium (ESUH) by Dr. Abdullah Shukur, at the University of Salahaddin, Erbil-Iraq. A voucher specimen was deposited with the accession number (7201).

Roots, leaves, and flowers were separately cleaned and air-dried in the shade at room temperature (20-25 ^{o}C). After drying, each part was grounded by using a laboratory grinding mill, to provide a homogeneous fine powder for the analysis. Powdered materials were stored in dark bottles and maintained at room temperature until required.

3.3. Extraction

T. systola roots, leaves and flowers (100g each) were separately defatted with *petroleum ether* (500mL), in an ultra-sonic bath for 30 min, then macerated for 3h under continuous stirring at room temperature. The procedure was repeated three times for each part. Defatted roots, leaves and flowers were subsequently separately extracted with ethanol (500mL) in an ultra-sonic bath for 30min, and then macerated for 3h under continuous stirring at room temperature. The procedure was repeated three times for each part. The mixtures were then filtered and the solvent removed under "vacuum" in a rotary evaporator to afford crude ethanol extracts: TR1 from roots, TF1 from flowers, and TL1 from leaves, respectively. After the ethanol extraction, the biomasses were separately extracted with 70% aqueous ethanol (500mL) in an ultra-sonic bath for 30min, and then macerated for 3h under continuous stirring at room temperature. The procedure was repeated three times for each part. The mixtures were then filtered and the solvent removed under "vacuum" to afford crude aqueous alcoholic extracts: TR2 from roots, TF2 from flowers and TL2 from leaves, respectively.

3.4. Spectrophotometric assays

3.4.1. Folin-Ciocalteau assay

The Folin-Ciocalteau reagent consists of a yellow solution of sodium tungstate ($Na_2WO_4{\cdot}H_2O$), sodium molybdate ($NaMoO_4{\cdot}H_2O$), lithium sulphate ($LiSO_4$), phosphoric acid (H_3PO_4) and hydrochloric acid (HCl).

Preparation of Reagents

- Folin-Ciocalteau reagent: 1 mL of reagent was diluted to 10 mL with deionized H_2O. The Folin -

Ciocalteau must be kept in a dark bottle in the shade.

- 20% (w / v) Na_2CO_3: 20g of Na_2CO_3 was dissolved in 100 mL of H_2O.
- 10% (v / v) EtOH: 10 mL of absolute EtOH was brought to 100mL with deionized H_2O.

Assay procedure

The reaction mixture consisted of a solution of the reference standard or extract in 10% EtOH (1mL), deionized H_2O (6 mL), and the Folin-Ciocalteau reagent (500 µL). The mixture was allowed to react for 3 min under stirring; then 1.5 ml of Na_2CO_3 (20% w / v) was added, and the mixture was diluted to totally 10 mL with deionized water. The flask was then sealed and stored in the dark for two hours at room temperature. The extracts of the plants were diluted as necessary to ensure that the final antioxidants concentrations fill within the range of the calibration curve of the standard. Absorbance values were determined at 760 nm, against a blank containing 10% EtOH (1 mL). The equation of the calibration curve was (y = 0.0785x + 0.0253) and the analyses were performed in duplicate. The total antioxidant content in each sample was calculated using the formula: C = c V/m where, C = total antioxidant content mg gallic acid equivalent (GAE)/g dry extract, c = concentration of gallic acid obtained from calibration curve in mg/mL, V = volume of extract in mL, m = mass of extract in gram. The total content of antioxidants in the sample was expressed as % gallic acid (w / w).[19]

3.4.2. Ammonium molybdate assay

Suitable working standards (1.0, 3.0, 5.0, 7.0 and 10 mg/mL) were prepared by dissolving the *Tulipa* extracts in distilled water. An aliquot of each solution (0.30 mL) was then mixed with 3 mL of the reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped with an aluminum foil, incubated at 95^0C for 90 min and then cooled to room temperature. The absorbance was measured at 695 nm against the blank. Ascorbic acid was used as the standard. Total antioxidant capacity was expressed as mg/mL equivalents of ascorbic acid. [8, 9]

3.4.3. DPPH radical scavenging activity

The ability of the *Tulipa* extracts to scavenge the DPPH free radical was assayed according to the method described by Shimada. [20]

A 0.1 mM solution of DPPH in MeOH was prepared. To 1 mL of this solution was added 4 mL of sample or extract solution in 40% aqueous MeOH at different concentrations (50-250 µg/mL). Subsequently, the mixture was shaken vigorously and incubated for 15min in the dark at room temperature until a stable absorbance value was obtained. The reduction of the DPPH radical was measured by continuously monitoring the decrease of the absorbance at 517 nm. In the control, samples were substituted by 40% aqueous MeOH. A lower absorbance of the reaction mixture indicated a higher free radical scavenging activity. The DPPH radical scavenging activity was calculated by the following equation:

$$\textbf{Scavenging (\%)} = (1\text{-}A_{sample517}/A_{control517}) \times 100$$

The IC_{50} value was the concentration of the sample required to scavenge 50% of the DPPH free radical. [8]

3.4.4. Fe (III) reducing power

The Fe (III) reducing power of *Tulipa* extracts was determined by the direct reduction of Fe (III) to Fe (II), by measuring the absorbance resulting from the formation of the Perl's Prussian blue complex following the addition of excess Fe^{3+} ions. This method is based on the reduction of potassium ferricyanide [Fe (III)] in stoichiometric excess relative to the antioxidants.

Extracts in different concentrations (500–900 µg/mL) in distilled water (0.75 mL) were mixed with 1.25 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 1.25 mL of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50 ^{0}C for 20 min, and then it was acidified with 1.25 mL of 10% trichloroacetic acid (TCA). Finally, 0.1% $FeCl_3$ (0.5 mL) was added, and the absorbance was measured at 700 nm. A higher absorbance of the reaction mixture indicates a higher reduction capability. [11, 21]

3.4.5. Hydrogen peroxide reducing activity

The hydrogen peroxide reducing assay was carried out following the procedure of Ruch and co-workers.[14] The principle of this method is based on the decrease of the H_2O_2 absorbance upon oxidation. A solution of 40 mM H_2O_2 in a 0.1 M sodium phosphate buffer (pH 7.4) was prepared. Subsequently, 250-450 µg/mL of *Tulipa* extract in 3.4 mL phosphate buffer was added to the H_2O_2 solution (0.6 mL), and the absorbance of the reaction mixture was recorded at 230 nm. The blank solution consisted of the sole sodium phosphate buffer. The concentration of H_2O_2 (mM) in the assay medium was determined using the following standard equation:

$$\mathbf{A_{sample230} = 0.505 \times [H_2O_2]}$$

The percentage of H_2O_2 reducing activity of *Tulipa* extracts and standard were calculated using the following equation:

$$\textbf{Reducing activity (\%)} = (1\text{- (Asample230/Acontrol 230)}) \times 100$$

Where $A_{control230}$ is the absorbance of the control and $A_{sample230}$ is the absorbance in the presence of *Tulipa* extract scavengers. [11].

3.4.6. Estimation of the Total Phenolic Content

The Total Phenolic Content was estimated spectrophotometrically with slight modifications, 0.1ml of a diluted

extract was further diluted to a final volume of 5ml with distilled water; subsequently, 5% $NaNO_2$ (0.3 mL) was added, followed by 10% $AlCl_3$ in distilled water (3 mL) 5 min later. After 6 min, 1 M NaOH (2 mL) was added and the absorbance was measured at 415 nm. Quercetin was used as the standard for constructing a calibration curve (y = 0.0368x + 0.0017), while the phenolic contents were expressed as mg quercetin equivalent/g of dry sample. Data were reported as the mean ± SD for duplicate measurements. [22-24]

4. Statistical analysis

Scatter and Bar Charts drawn by the Microsoft Excel 2010 and. IC_{50} values for all the experiments were determined by linear regression analysis through the equation y = (value) X + (value), where y is the 50% inhibition and X is the value of IC_{50} for each given y value. The data for all antioxidant activity tests are the average of duplicate analyses. The data were recorded as the mean ± SD.

5. Conclusion

We have evaluated for the first time the antioxidant and antiradical properties in vitro of extracts of roots, leaves, and flowers of *T. systola* Stapf., which grows wild on the mountains of the Kurdistan region of Iraq.

The significant antioxidant and antiradical activities determined for the different extracts give scientific support to the traditional use of the plant by the Kurdish peoples as a popular anti-inflammatory remedy and pain-relief and a great subtend to become a starting point for in vivo investigation in the next steps, isolation and characterization of the responsible antiradical and antioxidant secondary metabolites

Though the antioxidant and antiradical properties are undoubtedly due to the significant presence of phenolics in the different extracts (Fig. 3), a linear correlation between phenolic contents and the reducing activity was, however, not found. In fact, the roots resulted to contain the highest phenolic content (Fig. 3), while the ethanolic extract of flowers exhibited, in general, the highest antioxidant capacity in the assays in vitro. This result could be due to the presence, in the different parts of the plant, of phenolics, in particular flavonoids, with different redox properties, and/or the occurrence of antioxidants with structures different from phenols and displaying their action with different mechanisms. [15-18]

The study of the variety of secondary metabolites occurring in *T. systola* as a potential source for natural bioactive chemicals, as well as their precise antioxidant mechanisms, is therefore worthy of being carried out, and it will be reported in due time.

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