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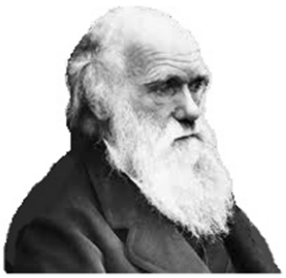

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A comparative study on the antioxidant efficiency of nine compounds commonly used as standards in antioxidant assays of extracts from medicinal plants and functional foods

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ABSTRACT

The detection of antioxidant activity in plant extracts or in pure compounds can be performed by a large number of methods with different reaction mechanisms, however, the criteria for choosing comparative standards are still not consensual. Thus, the present work intends to compare the antioxidant efficiency of nine substances, namely, gallic acid (GA), pyrogallol (PyG), propyl gallate (nPG), tannic acid (TA), quercetin (Qtn), rutin (Rut), ascorbic acid (Asc), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and butyl hydroxytoluene (BHT) using methods, (1) Ferric Reducing Antioxidant Power (FRAP) Assay, (2) Ferric Reducing Power (FRP), (3) Ferric-Ferrozine Antioxidant Capacity (FFAC), (4) Total Phosphomolybdenum Antioxidant Capacity (TAC) and (5) Radical Cation Elimination Assay 2,2' Azinobis(3 Ethylbenzothiazoline 6 Sulfonic Acid) (ABTS). Antioxidant efficacy by the 1,1-diphenyl-2-picrylhydrazine free radical scavenging method was previously described in a preliminary study. The results show that the maximum effectiveness was exhibited by PyG in the ABTS (0.425 ± 0.005 μM) and TAC (0.872 ± 0.075 μM) methods, Qtn in the FRP (5.776 ± 0.020 μM) and FFAC (20.390 ± 0.291 μM) methods and GA in the FRAP (6.765 ± 0.086 μM) and DPPH (1.105 ± 0.003 μM) methods. The results found in this study reveal that the effectiveness of a standard depends on the method applied, and the antioxidant activity of the same standard may present differences between the methods, which suggests that the selection of a comparative standard for the antioxidant activity tests of the extracts of plants or functional foods must be made according to the method to be applied.

***Keywords*:** Antioxidant, Gallic acid, Quercetin, Ascorbic acid, Pyrogallol

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1. INTRODUCTION

Several evidences reported in different literatures indicate a strong correlation between free radicals and the occurrence of several degenerative diseases, including diabetes, cancer and premature aging [1, 2].

Therefore, many nutritionists recommend the consumption of at least a minimum amount of food of plant origin (fruits or derivatives, vegetables and spices) or food supplements of natural and synthetic origin containing antioxidants to maintain the body's redox balance.

Because of this, research on medicinal plants and foods containing compounds with antioxidant power has currently become one of the most widespread topics in food and pharmaceutical sciences [3], as it is assumed that the consumption of products that contain this group of substances in their composition (polyphenols, ascorbic acid, tocopherols and carotenoids) can reverse the harmful effects of free radicals. Thus, several natural or synthetic compounds with antioxidant capacity have been widely investigated in recent years, due to their potential protective effects against oxidative stress (destruction of important biomolecules such as DNA, membrane lipids and proteins) [4, 5, 6].

The antioxidant characterization of these compounds present in these foods, beverages and supplements has been carried out using several models developed over the years as an important tool in the search for new substances or plant extracts whose antioxidant properties are not yet known [7, 8, 9, 10].

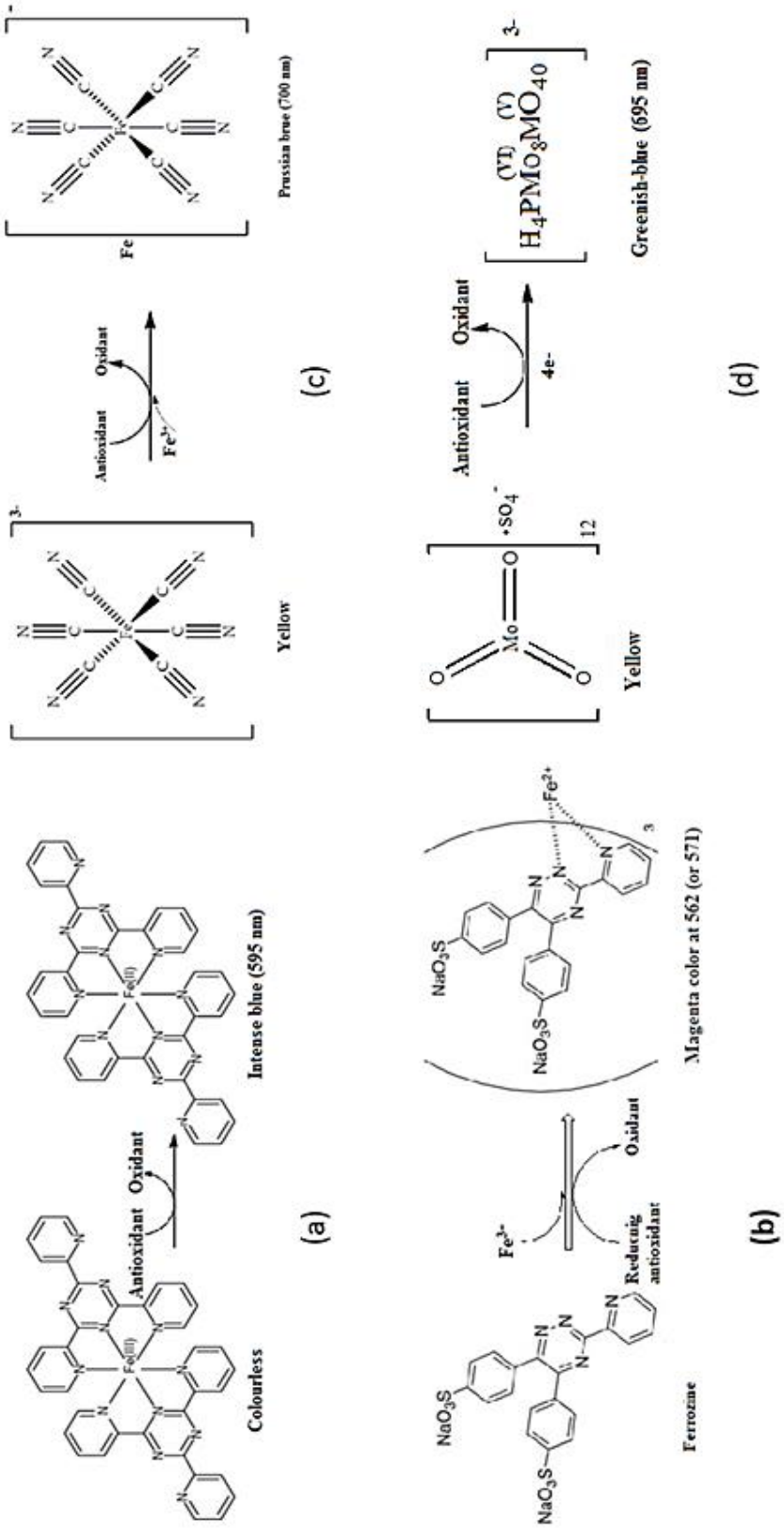
These methods can follow a variety of mechanisms, including hydrogen atom transfer, electron transfer, reducing power, and metal chelation. Therefore, understanding the main mechanisms, advantages and limitations of tests to measure antioxidant capacity is important for the right choice of methods to be used to achieve the closest result to reality [11]. No less important are the criteria for choosing the comparative standard substance, as sometimes the results of the same molecule can vary when different methods are applied [12].

This fact can lead to an erroneous attribution of the antioxidant efficacy of plant substances or extracts whose antioxidant potential is not yet known, as the use of standards with low efficacy for a given method can provide a false sense of high efficacy of the matrix under study [13].

Therefore, this scientific contribution can provide researchers with an important selective subsidy for defining antioxidant standards to be used depending on the method to be applied.

Thus, in the present study, the antioxidant efficiency of gallic acid (**GA**), pyrogallol (**PyG**), propyl gallate (**nPG**), tannic acid (**TA**), quercetin (**Qtn**), rutin (**Rut**), ascorbic acid (**Asc**), **Trolox** (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and butyl hydroxytoluene (**BHT**) were evaluated using the methods, (1) Ferric Reducing Antioxidant Power (FRAP) (Figure 1a), (2) Ferric-Ferrozine Antioxidant Capacity (FFAC) (Figure 1b), (3) Ferric Reducing Power (FRP) (Figure 1c), (4) Phosphomolybdenum Total Antioxidant Capacity (TAC) (Figure 1d) and (5) ABTS Radical Elimination (ABTS) (Figure 1e).

It should be noted that this work is the continuation of a project whose preliminary results found high antioxidant efficacy for GA and its derivatives (TA, PyG and nPG) in the DPPH free radical scavenging assay when compared to the other evaluated antioxidants [13].


Fe
Prussian brue (700 nm)
Antioxidant
Oxidant
Yellow
(c)
Antioxidant
Oxidant
4e-
Greenish-blue (695 nm)
Yellow
(d)
Intense blue (595 nm)
Antioxidant
Oxidant
Colourless
(a)
Magenta color at 562 (or 571)
Reducing antioxidant
Oxidant
Ferrozine
(b)

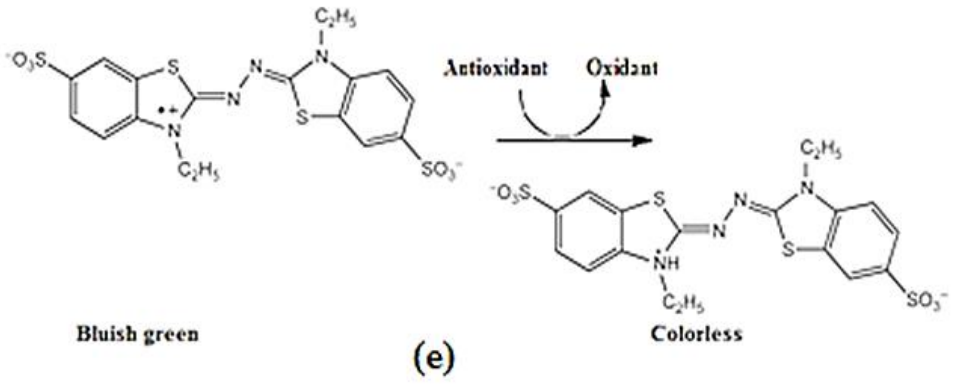


Figure 1(a-e). Mechanisms of the reactions used to determine the antioxidant capacity by the FRAP **(a)**, FFAC **(b)**, FRP **(c)**, TAC **(d)**, and ABTS **(e)** methods, respectively.

2. MATERIALS AND METHODS

2. 1. Chemicals and Equipment

Ethyl acetate (99.8%), Methanol (99.8%), Hydrochloric acid (32%) were provided by Ubuntu Chemical and Lab Supplies (Alberton, South Africa). Gallic acid (≥ 98%), Ascorbic acid (99.5%), Rutin (≥ 94%), Pyrogallol (≥ 98%), (±)-6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (97%), Trichloroacetic acid (≥ 99%), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt (97%), 2,2′-Azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt (≥ 98%), 2,4,6-Tris(2-pyridyl)-s-triazine (≥ 99%) were provided by Sigma Aldrich (Pty) Limited (Kempton Park, South Africa). Quercetin hydrate (95%) was provided by Acros Organics (New Jersey, USA). Tannic acid (88%) was provided by Sisco Research Laboratories Pvt. Ltd. (India, Maharashtra), Propyl gallate (99.5%) was provided by Panreac Química SA (Spain, Barcelona). Butylated hydroxytoluene (99.5%), was provided by Roig Farma, SA (Spain, Barcelona). Acetic Acid glacial *(99,8%)* was provided by Sky Chem & Lab Supplies (Alberton, South Africa). Centrifuge (Eppendorf 5804 R, Germany), Heating water bath (Buchi B-480, Switzerland) were used and spectrophotometric determinations were made using a spectrophotometer UV-VIS (Thermo Scientific Orion AquaMate 8000, USA).

2. 2. ABTS radical scavenging activity

In the ABTS assay, the green-blue radical cation, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) was produced by oxidation with potassium persulfate, with one of the maximum absorption zones of 734 nm. The antioxidant efficiency of GA, PyG, nPG, TA, Qtn, Rut, Asc, BHT, Trolox was measured by the ABTS radical scavenging method according to the procedure described by [14] with some modifications.

The ABTS radical was produced by reacting equal volumes of 2.45 mM potassium persulfate and 7 mM ABTS (dissolved in methanol). The resulting solution was incubated at room temperature for 16 hours in the dark before use. The ABTS solution was then diluted with methanol to obtain an absorbance of 0.700 ±0.005 at 734 nm. Then, 25 µL of the various samples duly diluted were added to 2.5 mL of the $ABTS^{+}$ solution and after 6 min of incubation

at room temperature, the absorbance was recorded at 734 nm using a UV/vis spectrophotometer (Thermo Scientific Orion AquaMate 8000, USA). All assays were done in three replicates and the percentage of $ABTS^+$ radical inhibition was calculated using equation 1.

$$\text{ABTS Inhibition (\%)} = \left(\frac{A_O - A_S}{A_0}\right) x100 \qquad (1)$$

where: *As*: Sample absorbance; *Ao*: Absorbance control;

2. 3. Ferric reducing power (FRP)

The reducing power of the samples was determined by the method described by [15] with some modifications. Briefly, 2.5 mL of each of the samples at different concentrations were mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of potassium ferricyanide (1%), the mixture was incubated at 50 °C (water bath) for 20 min. The reaction was stopped by adding 2.5 mL of 10% trichloroacetic acid (w/v) followed by centrifugation at 3000 rpm for 10 min. Finally, 2.5 mL of the supernatant was mixed with 2 mL of distilled water and 0.5 mL of ferric chloride (0.1%). The absorbance of the resulting solution was measured at 700 nm using a UV/vis spectrophotometer. All assays were done in three replicates and the percentage of iron reduction (FRP) was calculated using equation 2.

$$\text{FRP (\%)} = \left(1 - \frac{A_O - A_S}{A_0}\right) x100 \qquad (2)$$

2. 4. Ferric reducing antioxidant power (FRAP)

Determination of antioxidant activity by the FRAP assay was performed using the methodology described by [8] with minor modifications. For the assay, fresh FRAP reagent was prepared by mixing 75 mL of 300 mM acetate buffer, 7.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution and 7.5 mL of 20 mM of ferric chloride. For the assay, different concentrations of each sample (500 µL) were mixed with the FRAP solution (3.5 mL) and incubated in a water bath at 37 °C for 30 minutes. The readings of the colored solutions (ferrous tripyridyl triazine complex) formed were taken spectrophotometrically at 593 nm. All assays were performed in three replicates and the percentage of iron reduction was calculated using equation 2.

2. 5. Total phosphomolybdenum antioxidant capacity assay (TAC)

The determination of antioxidant activity by the Phosphomolybdenum Total Antioxidant Capacity assay was performed according to the procedure described by [10], with minor modifications. Briefly, 100 µL of each solution (in different concentrations) were mixed separately in test tubes, with 1 mL of phosphomolybdenum reagent (0.6 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and 1 mL of distilled water.

The tubes were covered with aluminum foil and incubated for 90 minutes at 90 °C, followed by cooling to room temperature. The absorbances of the greenish solutions produced were read at 695 nm, against an appropriate blank. All assays were performed in three replicates and the calculations of total antioxidant capacity (TAC) were performed using equation 2.

2. 6. Ferric-ferrozine antioxidant capacity (FFAC)

The antioxidant capacity of the samples, determined by the ferric-ferrozine assay, was performed using the method described by [16], with some modifications. Briefly, 5 mL of an aqueous solution containing 0.024 g of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ (molecular weight = 482.2) was mixed with 1 mL of 1 M HCl and 5 mL of an aqueous solution containing 0.123 g of ferrozine (weight molecular = 492.47). The mixture was diluted to 25 mL with distilled water to make the final iron (III) concentration equal to 2.0 mM and the ferrozine concentration 1.0 µM. For the assay, 1 mL of each sample (at different concentrations) was mixed with 20 µL of ferric-ferrozine solution and 3 mL of acetate buffer solution (2 mM, pH 5.5). The absorbance of the formed magenta solutions was measured at 562 nm against an appropriate blank after 10 min of incubation at room temperature. All assays were performed in three replicates and the results were expressed as mean ± standard deviation of the mean of the three replicates.

2. 7. Statistical analysis

Statistical treatment was performed using GraphPad Prism 9.0 Software. All results are expressed as mean ± standard deviation of the mean of three replicates. Differences between groups were detected using the t-student test and analysis of variance (one-way-ANOVA). Differences were considered significant for the value of $p < 0.05$.

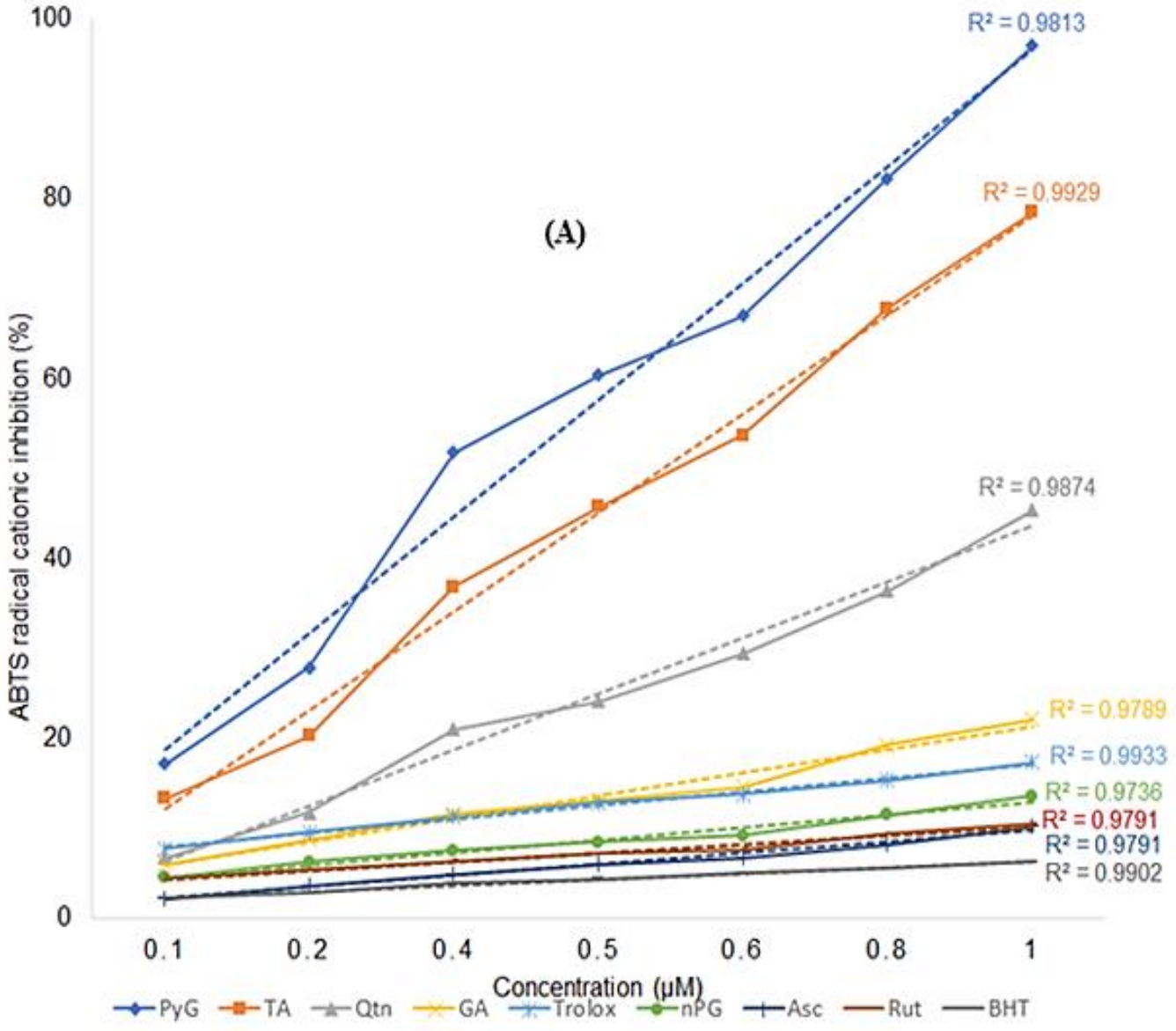
3. RESULTS AND DISCUSSION

Table 1. Antioxidant efficiency of standards for ABTS, FRP, FRAP, FFAC and TAC methods.

	EC_{50} (µM)				
Substance	**ABTS**	**FRP**	**FRAP**	**FFAC**	**TAC**
PyG	0.425 ± 0.005	5.919 ± 0.053	10.469 ± 0.128	87.665 ± 1.021	0.872 ± 0.075
TA	0.579 ± 0.007	6.222 ± 0.106	14.566 ± 0.211	67.947 ± 4.372[a]	10.578 ± 0.109
Qtn	1.125 ± 0.015	5.776 ± 0.020	8.205 ± 0.086	20.390 ± 0.291	21.636 ± 0.508
GA	2.574 ± 0.050	15.120 ± 0.178	6.765 ± 0.086	65.672 ± 1.494[a]	7.608 ± 0.186
Trolox	4.106 ± 0.015	97.351 ± 0.723	30.359 ± 0.834	41.571 ± 0.314	2.449 ± 0.037
nPG	4.805 ± 0.041	40.552 ± 0.981	11.667 ± 0.403	76.634 ± 1.612	74.649 ± 0.450
Asc	5.686 ± 0.081	91.048 ± 0.880	62.996 ± 0.505	33.279 ± 0.277	17.050 ± 0.113

Rut	6.951 ± 0.023	44.540 ± 0.341	9.926 ± 0.141	66.949 ± 1.463[a]	29.578 ± 0.219
BHT	10.772 ±0.113	70.173 ± 0.696	15.217 ± 0.157	323.402 ± 3.449	95.084 ± 0.846

Means with the same letter do not show significant differences for the value of $p < 0.05$. **PyG –** pyrogallol, **TA –** tannic acid, **Qtn –** quercetin, **GA –** gallic acid, **Trolox –** (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, **nPG –** n-propyl gallate, **Asc –** ascorbic acid, **Rut –** rutin and **BHT -** butylhydroxytoluene



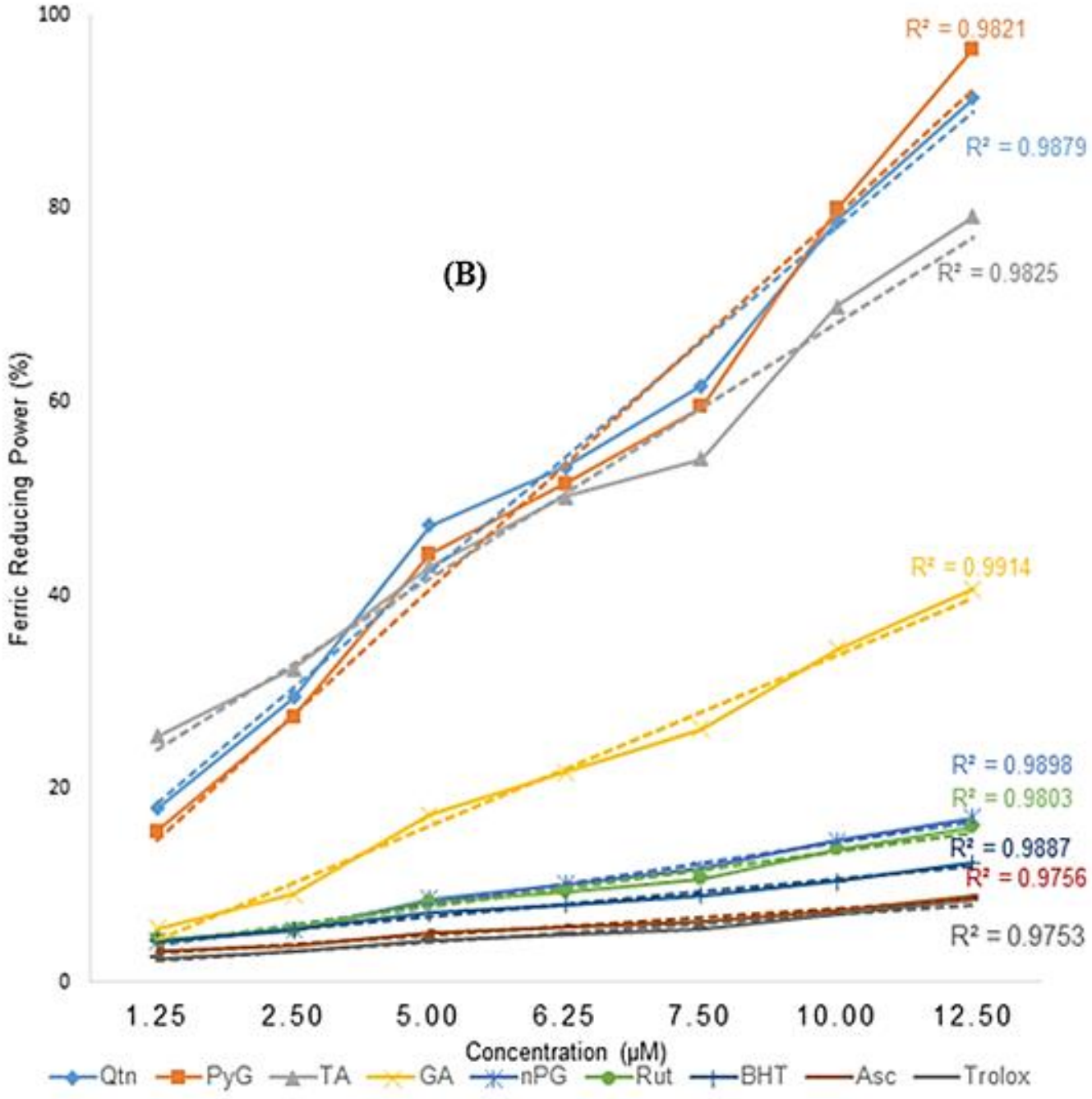
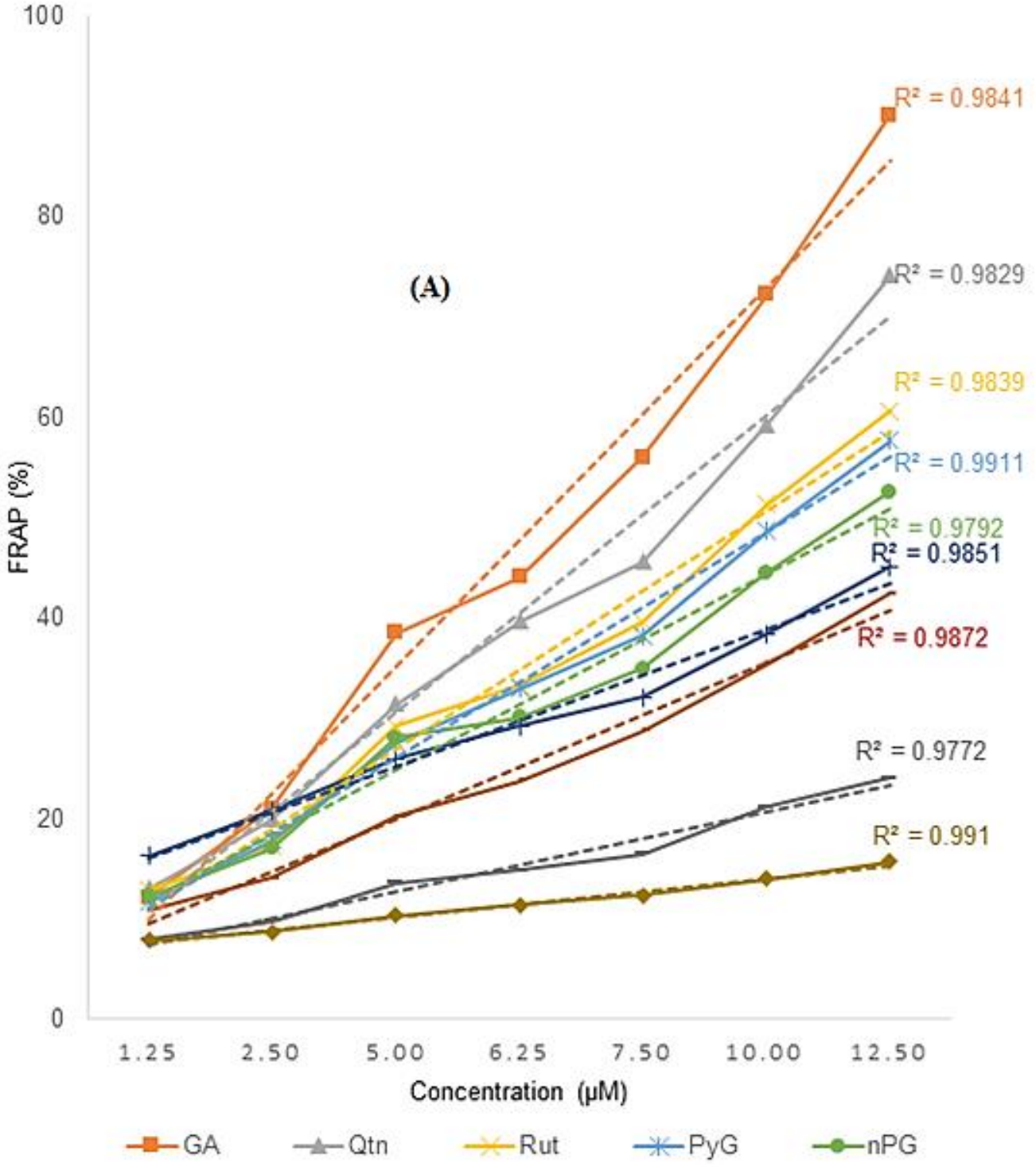
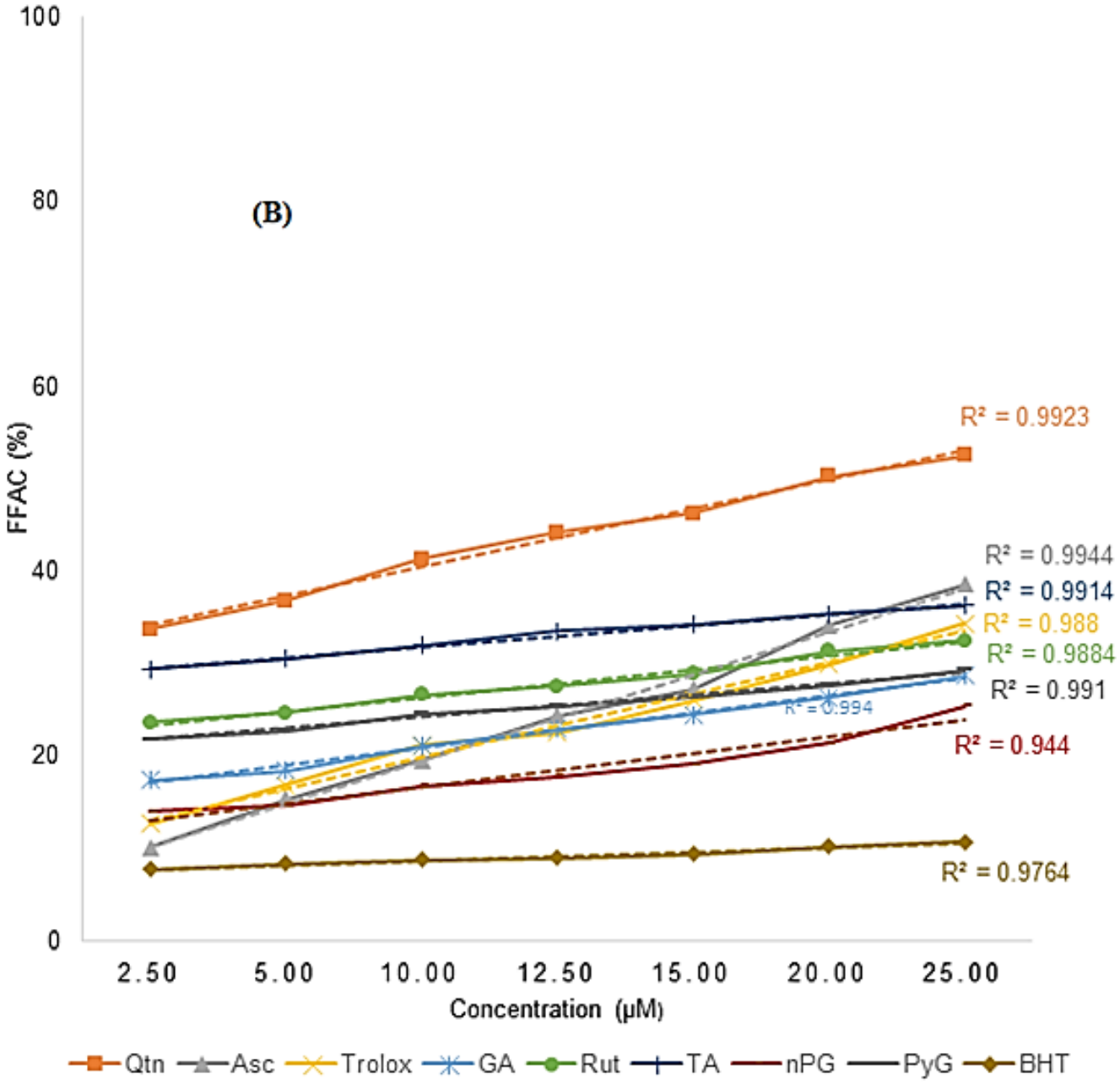


Figure 2. Dose (μM)-response (%) curves for ABTS **(A)** and FRP **(B)** methods.


100
80
60
40
20
0
FRAP (%)
(A)
R² = 0.9841
R² = 0.9829
R² = 0.9839
R² = 0.9911
R² = 0.9792
R² = 0.9851
R² = 0.9872
R² = 0.9772
R² = 0.991
1.25
2.50
5.00
6.25
7.50
10.00
12.50
Concentration (µM)
GA
Qtn
Rut
PyG
nPG


(B)
100
80
60
40
20
0
FFAC (%)
2.50
5.00
10.00
12.50
15.00
20.00
25.00
Concentration (μM)
R² = 0.9923
R² = 0.9944
R² = 0.9914
R² = 0.988
R² = 0.9884
R² = 0.991
R² = 0.994
R² = 0.944
R² = 0.9764
Qtn
Asc
Trolox
GA
Rut
TA
nPG
PyG
BHT

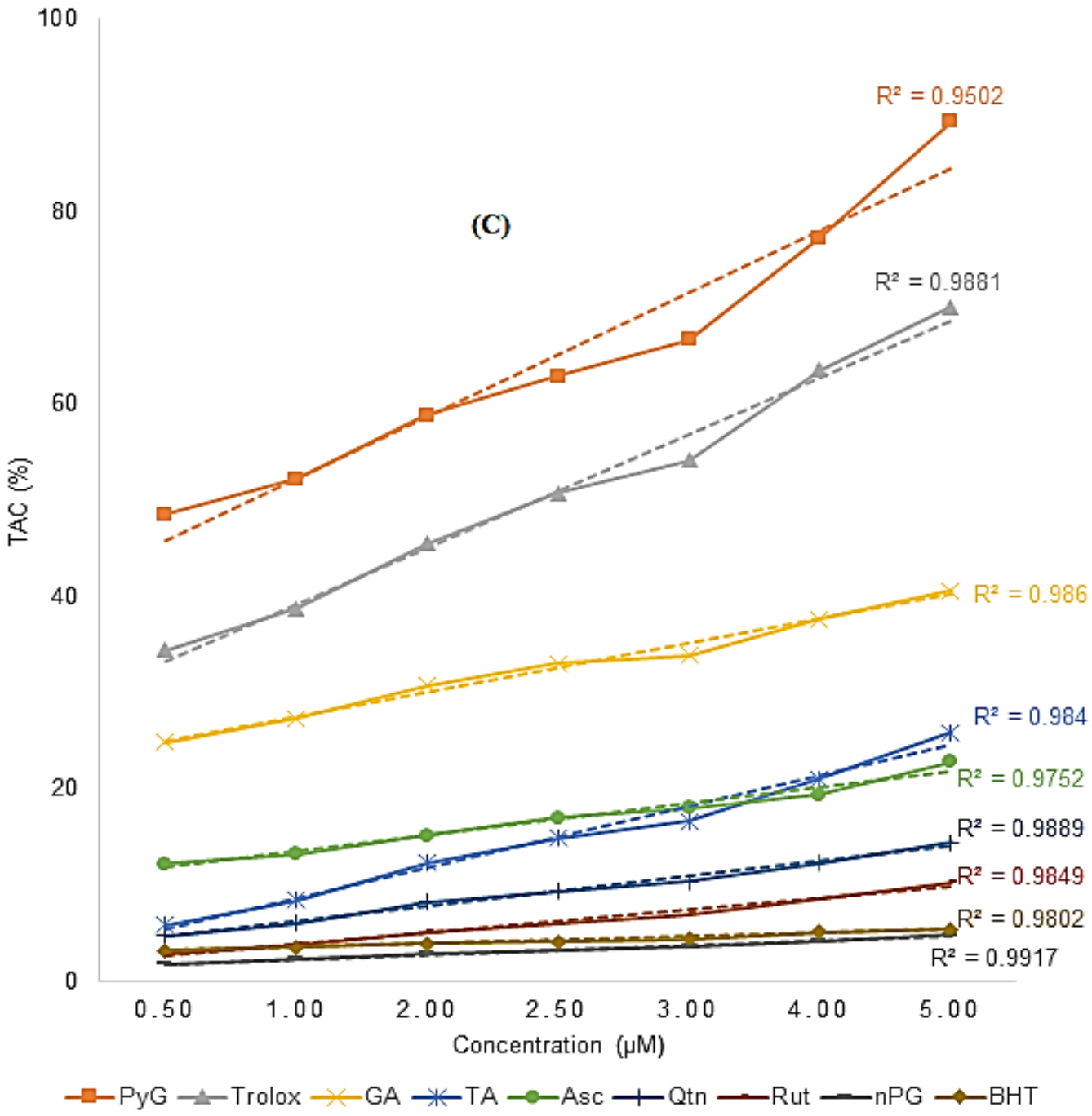


Figure 3. Dose (µM)-response (%) curves for FRAP **(A)**, FFAC **(B)** and TAC **(C)** methods.

In vitro tests of antioxidant capacity have become important instruments in the search for new bioactive substances, as well as in the analysis of already known ones. Therefore, several methods have been developed in recent years with the objective of being applied for this type of analysis, however, in the present work, the antioxidant capacity of the standards were

evaluated through five tests, that are, (1) ABTS cationic radical scavenging test (ABTS), ferric reducing power (FRP), ferric reducing antioxidant power (FRAP), ferric-ferrozine antioxidant capacity (FFAC), formation of the phosphomolybdenum complex (TAC). Table 1 shows the antioxidant efficiency results for the nine substances studied in efficient concentration values (EC_{50}), while Figures 2 and 3 show the dose-response graphs for the ABTS (Figure 2A), FRP (Figure 2B), FRAP (Figure 3A), FFAC (Figure 3B) and TAC (Figure 3C) methods, respectively.

3. 1. ABTS Radical Scavenging Assay

The ability to scavenge ABTS free radicals, described by the EC_{50} values (Table 1) for pure compounds, varies according to the order: PyG (0.425 ± 0.005 μM) > TA (0.579 ± 0.007 μM) > Qtn (1.125 ± 0.015 μM) > GA (2.574 ± 0.050 μM) > Trolox (4.106 ± 0.015 μM) > nPG (4.805 ± 0.041 μM) > Asc (5.686 ± 0.081 μM) > Rut (6.951 ± 0.023 μM) > BHT (10.772 ± 0.113 μM). The lowest EC_{50} value indicates a greater ability to scavenge free radicals from ABTS, which was demonstrated by PyG and Qtn, while Rut and BHT were less efficient when compared to the other studied antioxidants. These results differ slightly from those found by Cumbane et al. (2022) for the same substances in the DPPH free radical scavenging method, in that study, the antioxidant efficiency found, followed the following order: GA (1.110 ± 0.00 μM) > PyG (1.470 ± 0.00 μM) > TA (1.530 ± 0.01 μM) > nPG (4.640 ± 0.07 μM) > Qtn (5.770 ± 0.10 μM) > Asc (13.97 ± 0.16 μM) = Trolox (14.86 ± 0.09 μM) > Rut (14.92 ± 0.07 μM) > BHT (50.72 ± 0.11 μM). Note that GA and PyG were more efficient than the other antioxidants, however, for Rut and BHT, no differences were observed in the order of antioxidant efficiency in both methods. Figure 2A shows the concentration versus antioxidant activity relationship (dose-response curve), and the linear trend of all curves in the working concentration range (0.100 - 1.00 μM) is clearly seen.

3. 2. Ferric Reducing Power (FRP)

The FRP assay is based on the principle that substances with a reducing potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form a stable ferric-ferrous complex that has maximum absorption at 700 nm [17]. The FRP, described by EC_{50} values (Table 1) for the compounds under study varies in the order: Qtn (5.776 ± 0.020 μM) > PyG (5.919 ± 0.053 μM) > TA (6.222 ± 0.106 μM) > GA (15.120 ± 0.178 μM) > nPG (40.552 ± 0.981 μM) > Rut (44.540 ± 0.341 μM) > BHT (70.173 ± 0.696 μM). > Asc (91.048 ± 0.880 μM) > Trolox (97.351 ± 0.723 μM). The lowest EC_{50} value also indicates greater antioxidant capacity of the reducing substances, which was demonstrated by PyG and Qtn, while Trolox was less efficient when compared to the other studied antioxidants. These results also differ from those found by Cumbane et al. (2022) for the DPPH free radical scavenging method. Note that Qtn (flavonoid) occupied the fifth position for the DPPH method [13], second position for the ABTS method and first position for the FRP method in the order of antioxidant efficiency. These variations in the antioxidant efficiency of quercetin, as well as the other substances studied, support the idea that the antioxidant efficiency of a given substance may vary depending on the method applied [12]. However, this idea cannot be seen as universal, as, for BHT, these fluctuations were not observed in DPPH and ABTS methods. For Rut, in the DPPH and ABTS methods, no variations were found in the order of antioxidant efficiency, while in the FRP method, a significant increase in efficacy was observed,

which can be explained by the fact that the reaction medium of the method is slightly acidic, thus contributing to promote a partial or total hydrolysis of the glycosidic residue of this quercetin glycoside, which can lead to an increase in its antioxidant activity [18, 19]. Figure 2B shows the concentration versus activity antioxidant (dose-response curve), in this method a linear trend was also observed for all curves in the working concentration range (1.25 - 12.50 μM).

3. 3. Ferric Reducing Antioxidant Power (FRAP)

The antioxidant capacities of pure compounds against FRAP were measured at concentrations ranging from 1.25 to 12.50 μM, as represented in the graph of Figure 3A. The FRAP test takes advantage of electron transfer reactions and is based on the reduction of the ferric complex $[Fe(II)(TPTZ)_2]^{3+}$ (colorless) in the presence of reducing substances, in a strongly acidic medium (pH 3.6), producing the Prussian blue ferrous ion $[Fe(II)(TPTZ)^2]^{2+}$ [20, 21]. The redox potential of the $[Fe(II)(TPTZ)_2]Cl_3$ salt (~0.70 V) is comparable to that of the ABTS radical anion (~0.68 V). Therefore, essentially there is not much difference between the ABTS test and the FRAP test, differing only by the reaction conditions, as the ABTS test is carried out in neutral conditions, while the FRAP test is carried out in strongly acidic conditions [21]. Despite the apparent similarity, the results obtained for the two methods are not comparable, as for the FRAP method, GA (6.765 ± 0.086 μM), Qtn (8.205 ± 0.086 μM) and Rut (9.926 ± 0.141 μM) showed greater antioxidant activity in relation to the other substances studied (Table 1), while for ABTS, GA and Rut are not part of the series of the three most efficient substances in the group. However, these results corroborate with those observed by Schlesier et al. (2002) in which GA was the most efficient antioxidant for the FRAP method.

3. 4. Ferric-Ferrozine Antioxidant Capacity (FFAC)

Ferrozine is a highly stabilizing iron ligand, so the ferric ion in the presence of ferrozine easily oxidizes antioxidants and is reduced to Fe(II)-FZ, producing very high molar absorptivity and therefore increased sensitivity for most antioxidants. The ferric-ferrozine antioxidant capacity (FFAC), described by the EC_{50} values (Table 1) for the pure compounds varied in the order: Qtn (20.390 ± 0.291 μM) > Asc (33.279 ± 0.277 μM) > Trolox (41.571 ± 0.314 μM) > GA (65.672 ± 1.494 μM) = TA (67,947 ± 4,372 μM) = Rut (66,949 ± 1,463 μM) > nPG (76,634 ± 1,612 μM) > PyG (87,665 ± 1,021 μM) > BHT (323,402 ± 3,449 μM). The highest value of antioxidant activity (lower EC_{50}) by the ferric-ferrozine method was observed for Qtn (20.390 ± 0.291 μM), corroborating the result of the FRP assay, followed by Asc (33.279 ± 0.277 μM) and Trolox (41.571 ± 0.314 μM). GA, Rut, TA did not show significant variations in antioxidant activity among themselves. While the synthetic antioxidant BHT remained the least active of the bunch. The antioxidant capacity of the Qtn, GA and Trolox standards obtained by the FFAC method corroborate the results obtained by [16], in which Qtn showed greater antioxidant power compared to GA and Trolox. Figure 3B shows the concentration versus antioxidant activity, in this method a linear trend was also observed for all curves in the working concentration range.

3. 5. Total Phosphomolybdenum Antioxidant Capacity (TAC)

The TAC assay is based on the reduction of molybdenum (VI) to molybdenum (V) by the antioxidants present in the sample and subsequent formation of the green color of the phosphomolybdenum (V) complex at acidic pH [10]. The antioxidant capabilities of the pure

compounds against the phosphomolybdenum complex were measured at concentrations ranging from 0.50 - 5.00 μM as depicted in Figure 3C, showing a linear trend in this range of concentrations. The highest values of antioxidant activity were found in PyG (0.872 ± 0.075 μM), Trolox (2.449 ± 0.037 μM) and GA (7.608 ± 0.186 μM) samples, while BHT remains the least efficient antioxidant in the group (see Table 1).

4. CONCLUSIONS

With the results of the present study, it was possible to conclude that, in general, the antioxidant efficiency of a pure compound can vary depending on the method applied and, therefore, the choice of a comparative standard to measure the antioxidant capacity of plant extracts or foods should take into account its efficiency for the method to be applied.

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