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ORIGINAL RESEARCH PAPER

DEVELOPMENT OF SPECTROPHOTOMETRICAL ASSAY FOR THE STUDY OF THE INTERACTION OF ANTIOXIDANT STANDARDS WITH 1,1-DIPHENYL-2-PICRYLHYDRAZYL FREE RADICALS

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Abstract: The aim of the present study was to develop a spectrophotometric based assay for the determination of binding parameters like binding constant and binding free energy of the free and 1,1-diphenyl-2-picrylhydrazyl (DPPH) bound forms of a set of antioxidant standards (AS). The determination of binding constant and binding free energy is based upon the decrease in absorbance of the electronic absorption spectrum of a 10^{-4} M acetonitrile solution of DPPH in the presence of a given concentration of a solution of antioxidant standards in the same solvent.

Keywords: *binding constants, free energy, UV-Vis*

INTRODUCTION

Scientific literature dealing with the study of the antioxidant activity of phenolic compounds and antioxidant standards, generally focuses on evaluating the antioxidant activity using different methods based on scavenging activity of free radicals like 1,1-diphenyl-2-picrylhydrazyl (DPPH) [1 – 5] and superoxide anion ($O_2^{\cdot-}$) [6 - 10]. Information on binding parameters of DPPH with antioxidant standards or with potential antioxidant compounds have never, as far as we know, been reported until now.

The aim of this work is to provide a new method, based on spectrophotometric measurements, for the determination of binding parameters of a set of four commonly used antioxidant standards with DPPH radical.

The decrease in absorbance of an acetonitrile solution of DPPH in the presence of antioxidant standards were used to investigate the interaction between DPPH and antioxidant standards.

MATERIALS AND METHODS

Chemical

Acetonitrile (ACN) (HPLC-grade from Sigma-Aldrich) was used as solvent without further purification, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (99%), ascorbic acid (99.7 %) (AA), gallic acid (99 %) (GA), caffeic acid (99 %) (CA), rutin (97 %) (R) were all purchased from Alfa Aesar and used without further purification. All other reagents used are analytical grade.

Instrumentation and software

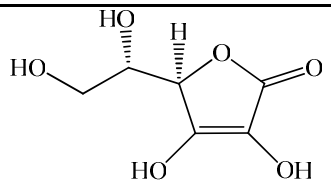
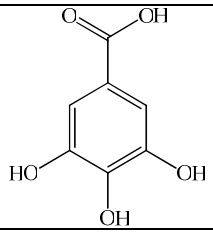
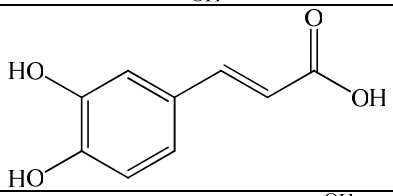
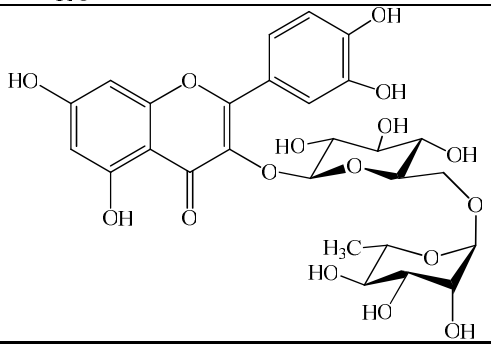
UV-Vis experiments were performed using a UV-Vis spectrometer (Shimadzu 1800) and a quartz voltammetric cell with a volumetric capacity of 5 mL. Data acquisition was accomplished with a Pentium IV (CPU 4.0 GHz and RAM 2 Gb) microcomputer using UV probe software version 2.34 (Shimadzu). Graphs plot and calculus were carried out using OriginLab software version 2.0 (Integral Software, France).

The electronic spectrum of 10^{-4} M of DPPH in acetonitrile was obtained without standard antioxidants. The spectroscopic response of the same amount of DPPH was then measured after the addition of gradually increasing concentration of a solution of the standard antioxidant in the same solvent.

Antioxidant standards

Among several commonly used antioxidant standards, four are chosen for evaluation of their binding parameters with 1,1-diphenyl-2-picrylhydrazyl free radicals (Table 1).

Table 1. *Antioxidant standards (AS) used in the present study*

Molecular structure	Nomenclature
	*Common name*: **Ascorbic acid** *IUPAC name*: 1,2-dihydroxyethyl-3,4-dihydroxyfuran-2(5H)-one *Formula*: $C_6H_8O_6$
	*Common name:* **Gallic acid** *IUPAC name*: 3,4,5-trihydroxybenzoic acid *Formula:* $C_7H_6O_5$
	*Common name:* **Caffeic acid** *IUPAC name:* 3-(3,4-dihydroxyphenyl)-2-propenoic acid *Formula:* $C_9H_8O_4$
	*Common name:* **Rutin** *IUPAC name:* 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-{[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-({[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy}methyl)oxan-2-yl]oxy}-4H-chromen-4-one *Formula:* $C_{27}H_{30}O_{16}$

RESULTS AND DISCUSSION

Spectrophotometric studies of DPPH-AS interaction

The decrease in absorbance of DPPH in the presence of antioxidant standards can be used for the calculation of the binding constant and the binding free energy, whereas the shift in wavelength values can be exploited for the determination of the mode of interaction, these observations are inspired from the study of the binding of drug molecules to DNA [11 – 14].

The interaction of AS with DPPH was studied by UV-Vis absorption titration for getting further clues about the mode of interaction and binding strength. The effect of different concentration of AS on the electronic absorption spectrum of a 10^{-4} M solution of DPPH in acetonitrile is shown in Figures 1, 2, 3, and 4. It was observed that DPPH had one strong absorption peak at 518 nm. After interaction with increasing amount of antioxidant standards, this peak decreased gradually and the wavelength had no obvious shift. The concentrations of antioxidants standards were chosen based on the optimal response in the electronic absorption spectrum.

Ascorbic acid-DPPH interaction

Figure 1 shows the electronic spectra of DPPH interaction with different concentrations of ascorbic acid.

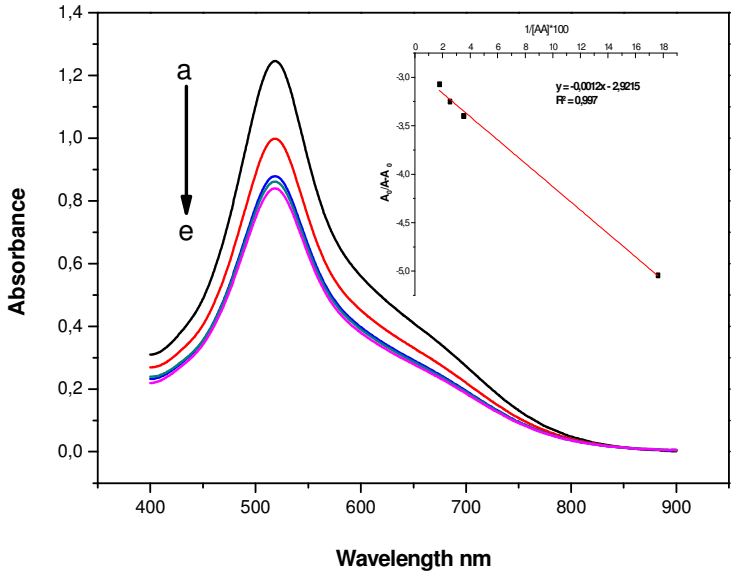


Figure 1. *Electronic absorption spectra of 10^{-4} M DPPH interaction with 0 (a), 5.6×10^{-4} M (b), 2.8×10^{-3} M (c), 3.9×10^{-3} M (d), 5.6×10^{-2} M (e), AA in ACN, Inset: plot of $1/[AA]$ versus $A/A_0 - A$*

Gallic acid-DPPH interaction

Figure 2 shows the UV spectra of DPPH interaction with different concentrations of gallic acid.

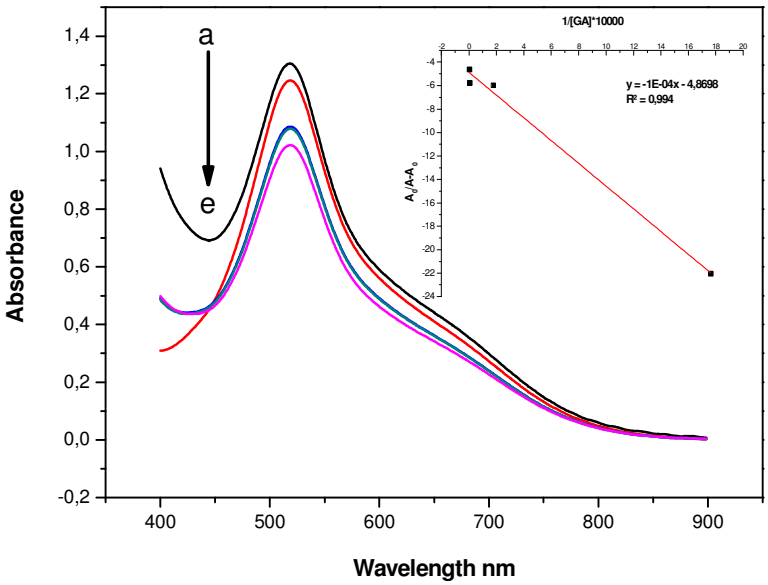


Figure 2. *Electronic absorption spectra of 10^{-4} M DPPH interaction with 0 (a), 5.6×10^{-8} M (b), 2.8×10^{-6} M (c), 2.8×10^{-3} M (d), 4.5×10^{-3} M (e), GA in ACN, Inset: plot of $1/[GA]$ versus $A/A_0 - A$*

Caffeic acid-DPPH interaction

Figure 3 shows the UV spectra of DPPH interaction with different concentrations of caffeic acid.

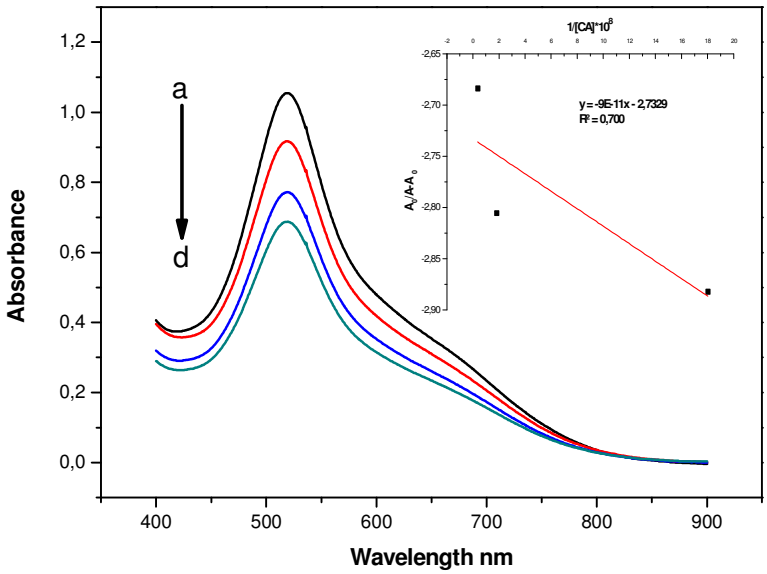


Figure 3. *Electronic absorption spectra of 10^{-4} M DPPH interaction with 0 (a), 5.5×10^{-7} M (b), 5.5×10^{-6} M (c), 2.8×10^{-2} M (d), CA in ACN, Inset: plot of 1/[CA] versus $A/A_0 - A$*

Figure 4 shows the UV spectra of DPPH interaction with different concentrations of rutin.

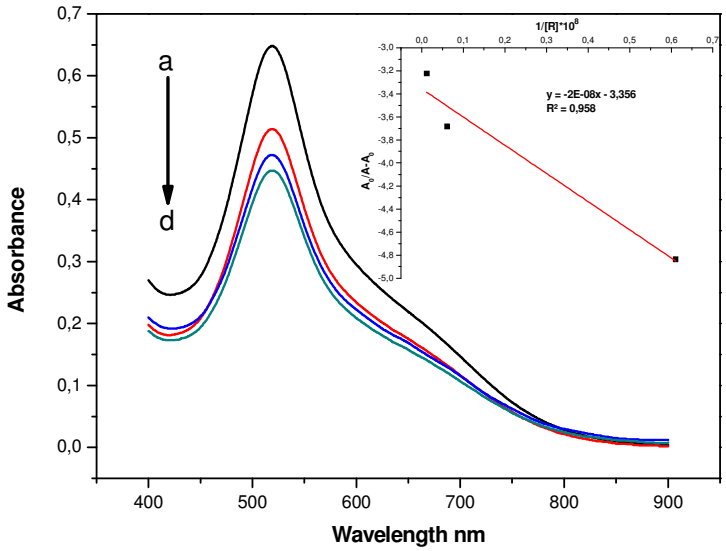


Figure 4. *Electronic absorption spectra of 10^{-4} M DPPH interaction with 0 (a), 1.6×10^{-5} M (b), 1.6×10^{-4} M (c), 8.1×10^{-4} M (d), R in ACN,*

Inset: plot of $1/[R]$ *versus* $A/A_0 - A$

Binding constant

The addition of gradually increasing concentration of AS in acetonitrile to a solution of 10^{-4} M of DPPH in the same solvent causes a remarkable overall decrease in absorbance (Figures 1, 2, 3 and 4). Based upon this decrease in absorbance, the binding constant was calculated according to Benesi-Hildebrand equation (1) used for the determination of the intrinsic binding constant/association constant of anticancer drug with DNA [15].

$$\frac{A_0}{A - A_0} = \frac{\varepsilon_0}{\varepsilon - \varepsilon_0} + \frac{\varepsilon_0}{\varepsilon - \varepsilon_0}\frac{1}{K[AS]} \quad (1)$$

where K is the binding constant, A_0 and A are absorbance of DPPH in the absence and in the presence of antioxidant standards, ε_0 and ε are their absorption coefficients respectively, $[AS]$ concentration of antioxidant standard.

The slope to intercept ratio of the plot between $A_0/(A - A_0)$ versus $1/[AS]$ yielded the binding constants, results are regrouped in Table 2. The moderate binding constants of ascorbic and gallic acids are indicative of electrostatic interaction, however higher values of binding constants of caffeic acid and Rutin are indicative of chemical interaction. The negative values of the Gibbs energy change signify the spontaneity of DPPH-AS interaction.

Table 2. *Binding constants and binding free energy values*

Compound	Equation	R^2	K [L·mol^{-1}]	ΔG [KJ·mol^{-1}]
DPPH-AA	$y = -1.2\times10^{-3}x - 2.9215$	0.997	24.3×10^{2}	-19.29
DPPH-GA	$y = -1.0\times10^{-4}x - 4.8698$	0.994	48.7×10^{3}	-26.70
DPPH-CA	$y = -9.0\times10^{-11}x - 2.7329$	0.700	30.4×10^{9}	-59.70
DPPH-R	$y = -2.0\times10^{-8}x - 3.3560$	0.958	16.8×10^{7}	-46.84

CONCLUSION

The binding parameters like binding constant and binding free energy of four commonly used antioxidant standards with 1,1-diphenyl-2-picrylhydrazyl (DPPH), were successfully determined using spectrophotometric based assay. The results indicated electrostatic interaction of DPPH radical with antioxidant standard ascorbic and gallic acid as the dominant mode, whereas caffeic acid and rutin interact chemically with DPPH.

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