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SPECTROPHOTOMETRICAL STUDY OF ANTIOXIDANT STANDARDS INTERACTING WITH 2,2-DIPHENYL-1-PICRYLHYDRAZYL RADICAL

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Abstract: This work aims to study the interaction of four well known antioxidant standards with 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) using spectrophotometric assays. The binding parameters like binding constant and binding free energy of the free and DPPH$^{\bullet}$ bound forms were determined. The determination is based upon the decrease in absorbance of the electronic absorption spectrum of an acetonitrile solution of DPPH$^{\bullet}$ in the presence of gradually increasing amount of antioxidant standards.

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

1. Introduction

Recently, attention has more been focused on evaluation of antioxidant activity using different techniques and assays based on scavenging activity of free radicals like 2,2-diphenyl-1-picrylhydrazyl and superoxide anion [1-13]. In addition, a wide variety of *in vitro* methods to assess radical scavenging ability and antioxidant activity was recently of great interest. Antioxidant activity is widely used as a parameter for medicinal bioactive components [14-16].

Latter, a few studies on the determination of binding parameters of DPPH$^{\bullet}$ with antioxidant standards or with potential antioxidant compounds have been reported [17].

In this study we investigate the antioxidant activity, the scavenging proprieties and the interaction of DPPH$^{\bullet}$ with four different antioxidant standards (AS) (α-tocopherol (Toc), catechin (Cat), butylated hydroxyanisole (BHA), querstine (Que). The choice of these four antioxidant standards is based on their decreasing antioxidant activity. The method used in this study is based upon the decrease in absorbance of an acetonitrile solution of DPPH$^{\bullet}$ in the presence of an antioxidant standard.

2. Experimental

2.1. Materials

Acetonitrile (ACN) (HPLC-grade from Sigma-Aldrich) was used as solvent without further purification, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) (99 %), α-tocopherol (99.7 %), catechin (99 %), BHA (> 99 %), querstine (99 %), were all purchased from Alfa Aesar and used without further purification. All other reagents used are of analytical grade.

2.2. Instruments

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$^{\bullet}$ solution in acetonitrile was obtained without standard antioxidants. The spectroscopic response of the same solution of DPPH$^{\bullet}$ was then measured after the addition of gradually increasing concentration of a solution of the standard antioxidant in the same solvent.

2.3. Evaluation of Antioxidant Activity

The antioxidant activity of standards was measured using a spectrophotometric method based on UV-Vis absorption spectroscopy techniques. The violet solution of the stable radical DPPH$^{\bullet}$ absorbs at 517 nm, but upon reduction by an antioxidant or a radical species the colour of the initial solution turns into yellow and its absorption decreases [18]. Briefly, 1 ml of 0.25 mM solution of DPPH$^{\bullet}$ was prepared in acetonitrile and 0.1 ml of various concentrations of AS solution in the same solvent was

added. The contents were mixed thoroughly and incubated for 30 min in dark at room temperature (301 ± 1 K).

The degree of reduction of absorbance was measured at 517 nm against blank samples. Lower absorbance of the reaction mixture indicates higher DPPH$^{\bullet}$ free radical scavenging activity. A standard curve was plotted using different concentrations of DPPH$^{\bullet}$.

The ability of the test sample to quench DPPH$^{\bullet}$ (% inhibition of DPPH) was calculated using Eq. (1):

$$\%\,\text{DPPH}^{\cdot}\ \text{scavenging activity} = \frac{A_c - A_s}{A_c} \times 100 \qquad (1)$$

where A_c is the absorbance of control without AS and A_s is the absorbance of sample. The DPPH$^{\bullet}$ concentration scavenging activity was expressed as mg/ml in the reaction medium and calculated from the calibration curve determined by linear regression.

2.4. Evaluation of Interaction

UV-Visible absorption spectroscopy was used to evaluate the interaction of DPPH$^{\bullet}$ with antioxidant standards, it is may be the most frequently used instrumental technique for studying both the antioxidant activity of potential antioxidant compounds and their interactions with DPPH$^{\bullet}$. The study of interactions DPPH$^{\bullet}$−AS could be carried out using spectrophotometrical techniques by checking the changes in the absorption peak of the DPPH$^{\bullet}$. Usually, DPPH$^{\bullet}$ molecules show one strong absorption peak that can be clearly distinguished in the visible region at 517 nm. To determine whether there is any interaction between the DPPH$^{\bullet}$ and AS is to compare the height of this peak from when the DPPH$^{\bullet}$ is free in solution to when the DPPH$^{\bullet}$ is bound with the AS. It has been assumed that rapid decreasing could be interpreted as an indication of the strength of the interaction between the DPPH$^{\bullet}$ and the AS molecules. The magnitude of the shifting of the position of the maximum of the peak can also be used as an indication of the strength of the interaction [17].

3. Results and Discussion

3.1. DPPH$^{\bullet}$ Scavenging Activity

In order to express the antioxidant activity of different antioxidant standards and to obtain kinetic curves from which IC_{50} values can be calculated, the radical scavenging activity of DPPH$^{\bullet}$ was plotted against different standards concentrations (data are not presented).The antioxidant activity of standards was expressed as IC_{50}. The IC_{50} values were defined as the concentration in milligrams per millilitre of samples that inhibits the formation of DPPH$^{\bullet}$ by 50 %. The equations obtained from the linear calibration graph in the studied concentration range for standards are summarized in Table 1 (where *y* represents the values of absorbance and *x*, the values of samples concentration, expressed as mg/ml).

Table 1

IC_{50} values of standards obtained using DPPH$^{\bullet}$ scavenging activity

Compound	Equation	R^2 values	IC_{50}, mg/ml
Toc	$y = 389.7x + 22.42$	0.922	0.07077
Cat	$y = 81020x + 35.66$	0.989	0.00018
BHA	$y = 31913x + 24.66$	0.862	0.00079
Que	$y = 15.42x + 36.34$	0.999	0.88586

3.2. Spectrophotometric Studies of DPPH$^{\bullet}$−AS Interaction

Following the same methodology as described for the study of the binding of drug molecules to DNA [11, 14-16], the decline of the electronic absorption spectrum of an acetonitrile solution of DPPH$^{\bullet}$ in the presence of a solution of AS at different concentrations in the same solvent was exploited for the calculation of the binding constant and the binding free energy of the product DPPH$^{\bullet}$−AS. In addition, if it is the case, the shift in wavelength values can be exploited for the determination of the mode of interaction.

The effect of different concentrations of AS on the electronic absorption spectrum of 10^{-4} M solution of DPPH$^{\bullet}$ in acetonitrile is shown in Fig. 1. All electronic spectrums showed one strong absorption peak at 517 nm. This peak decreased gradually after addition of an increasing amount of AS, which indicated interaction of DPPH$^{\bullet}$ with AS. The wavelength had no obvious shift. It has been noticed that in some case the absorbance of DPPH is increased with the addition of AS. The increase in absorbance is probably due to the interference of the absorption of the DPPH-AS adduct at 517 nm.

3.3. Determination of Binding Constants

The addition of gradually increasing amount of SA to a solution of 10^{-4} M of DPPH$^{\bullet}$ led to a notable decrease of the electronic absorption spectrum (Fig. 2). This decrease in absorbance was exploited for the calculation of binding constants and binding free energy using Benesi-Hildebrand equation (2) used for the determination of the binding constants of anticancer drug with DNA [17].

$$\frac{A_0}{A - A_0} = \frac{e_0}{e - e_0} + \frac{e_0}{e - e_0}\frac{1}{K[AS]} \qquad (2)$$

In Eq. (2), K refers to the binding constant, A_0 and A are absorbance of DPPH$^{\bullet}$ in the absence and in the presence of antioxidant standards, respectively, ε_0 and ε are their absorption coefficients, [AS] concentration of antioxidant standard.

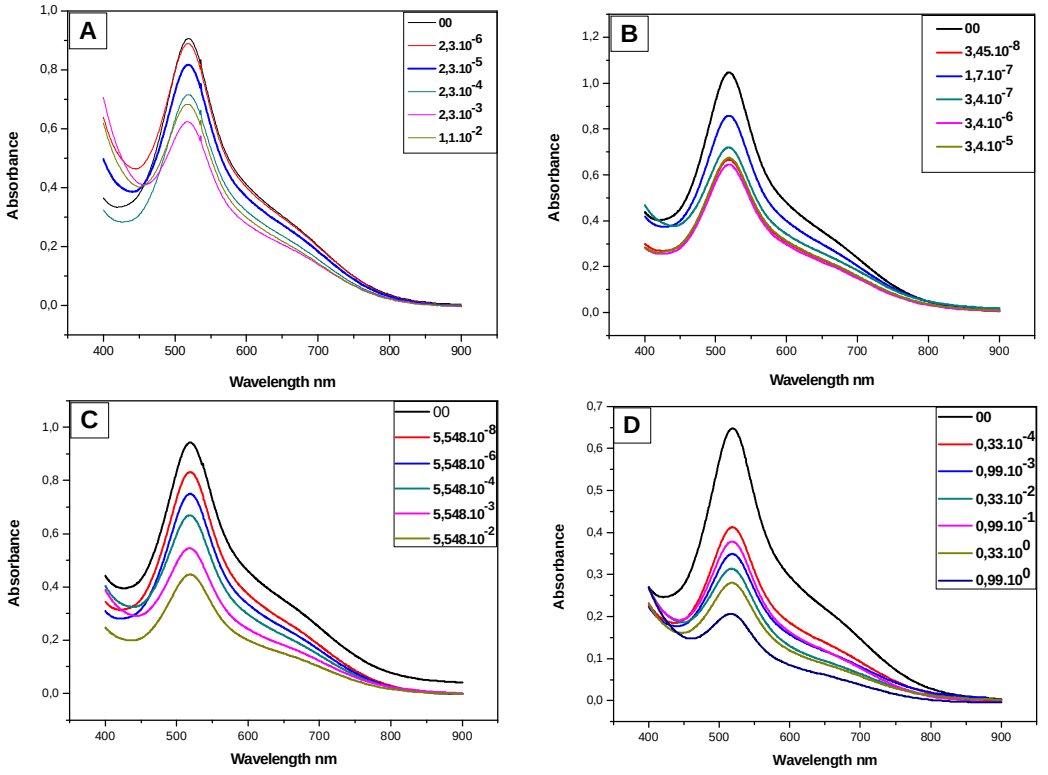

Fig. 1. Electronic absorption spectra of 10^{-4} M DPPH interaction with α-tocopherol (a), catechin (b), BHA (c), querstine (d) in CAN

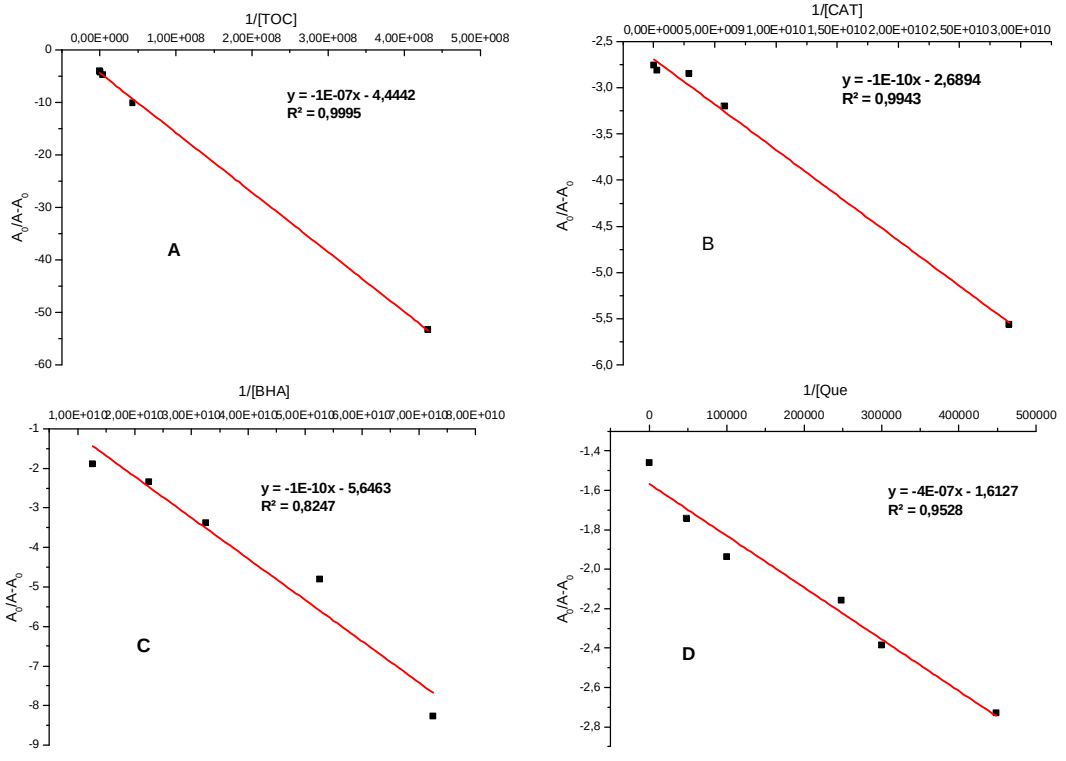

Fig. 2. (1/[AS] *vs.* $A_0/A\text{-}A_0$ for DPPH• with varying concentration of TOC (a), CAT (b), BHA (c) and QUE (d) used to calculate the binding constants of $DPPH^{\bullet}$ – AS products

Table 2

Binding constants and binding free energy values

Compound	Equation	R^2	K, l·mol^{-1}	ΔG, KJ.mol^{-1}
DPPH – TOC	$y = -1{\cdot}10^{-7}x - 4.4442$	0.999	$44.4{\cdot}10^{6}$	-43.55
DPPH – CAT	$y = -1{\cdot}10^{-10}x - 2.6894$	0.994	$26.89{\cdot}10^{9}$	-59.40
DPPH – BHA	$y = -1{\cdot}10^{-10}x - 5.6463$	0,825	$56.46{\cdot}10^{9}$	-61.23
DPPH – QUE	$y = -4{\cdot}10^{-7}x - 1.6127$	0,953	$40.31{\cdot}10^{5}$	-37.62

The ratio slope/intercept of the plot $A_0/(A-A_0)$ *vs.* 1/[AS] gave the binding constants, values are summarised in Table 2. The lower binding constants values of antioxidants α-tocopherol and querstine are suggestive of physical electrostatic interaction, whereas higher values of binding constants of catechin and BHA are indicative of chemical interaction [19]. The negative values of the Gibbs energy change ($\Delta G = -RT \ln K$) indicate the spontaneity of DPPH$^{\bullet}$ – AS interaction.

3.4. Correlation between Antioxidant Activity and Binding Free Energy

A direct correlation between antioxidant activities and binding free energy was demonstrated by nonlinear fitting analysis. The two parameters showed a high correlation coefficient of $r^2 = 1$ (Fig. 3).

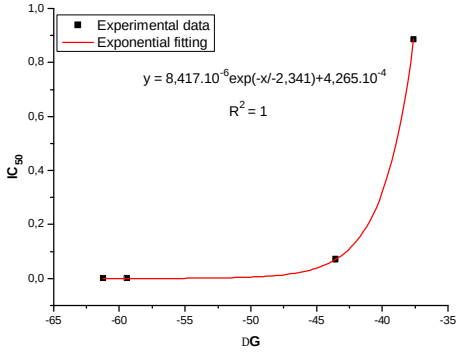

Fig. 3. Correlation of antioxidant activity and binding free energy

4. Conclusions

According to data obtained from the present *in vitro* study, all studied antioxidant standards interact spontaneously with 2,2-diphenyl-1-picrylhydrazyl radical. Catechin and BHA were found to interact chemically to DPPH$^{\bullet}$, whereas α-tocopherol and querstine undergo electrostatic interaction. Correlation of antioxidant activities and binding free energy shows moderate linearity, which demonstrates that the magnitude of binding free energy has a moderate contribution to the total antioxidant activity.

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СПЕКТРОФОТОМЕТРИЧНІ ДОСЛІДЖЕННЯ ВЗАЄМОДІЇ АНТИОКИСНЮВАЛЬНИХ СТАНДАРТІВ З РАДИКАЛОМ 2,2-ДИФЕНІЛ-1-ПІКРИЛГІДРАЗИЛ

Анотація. *З використанням спектрофотометричних методів аналізу вивчено взаємодію чотирьох добре відомих антиокиснювальних стандартів з радикалом 2,2-дифеніл-1-пікрилгідразил (ДФПГ). Визначено параметри зв'язування, такі як константа зв'язування та вільної енергії зв'язування вільних та зв'язаних форм ДФПГ. Визначення ґрунтується на зниженні поглинання електронного спектра в розчині ацетонітрилу ДФПГ у присутності поступово зростаючої кількості антиокиснювальних стандартів.*

Ключові слова: *константа зв'язування, вільна енергія, УФ-спектроскопія.*