

New approach for measuring antioxidant activity via graphite sensor

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ABSTRACT

An ion selective membrane sensor from dioctyl phthalate as a plasticizer in a polymeric matrix of polyvinyl chloride (PVC) and β -cyclodextrin as an ionophore was constructed and evaluated according to IUPAC recommendations. Linear Nernstian response of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH[•]) within the concentration ranges of 10^{-6} to 10^{-2} mol L⁻¹ was obtained with average recovery 99.87 ± 0.617 . Nernstian slope of 58.5 mV/decade with excellent selectivity over the pH range of 3–8 was observed. The suggested method was standardized using butylated hydroxyl anisole (BHA). The 50% radical scavenging activity (IC₅₀) determined by the proposed sensor correlated well with that of the common colorimetric method based on scavenging of DPPH[•]. An algorithm implemented in Microsoft Visual Basic[®] 6.0 was used for calculating (IC₅₀) values which are 7.38 μ g/ml \pm 0.35, 89.98 μ g/ml \pm 0.45 and 1.45 mg/ml \pm 1.50 for BHA, paracetamol and dipyridamole, respectively. The proposed sensor represents a simple and reproducible tool for measuring DPPH[•] scavenging activity of paracetamol and dipyridamole in bulk powder, pharmaceutical formulations and simulated intestinal fluid (SIF) without sophisticated separation techniques.

INTRODUCTION

The chemistry of free radicals and antioxidants is of great importance, particularly in the areas of clinical medicine and nutritional science (Halliwell and Gutteridge, 1999). Antioxidants act as free radical scavengers and can prevent the damage caused by oxidative reactions, including cancer, Alzheimer's and Parkinson's diseases (Balazs and Leon, 1994). Hence, the evaluation of the antioxidative activity of medical, cosmetic and food samples provides useful clinical information (Lester and Helmut, 2007).

One of the most commonly used methods for in vitro evaluation of antioxidant capacity is DPPH[•] scavenging method (Magalhães *et al.*, 2008c; Gu'lcin, 2006a; Gourine *et al.*, 2010; Beara *et al.*, 2012; Tadhani, 2007; Blois, 1958). Recently, A number of DPPH[•]-based tests for the assessment of antioxidant activity have been developed. The evaluation of antioxidant capacity based on the amperometric reduction of DPPH[•] at the glassy carbon electrode was reported (Milardovic *et al.*, 2006a; Milardovic *et al.*, 2005b). TLC (Cies'la *et al.*, 2012), HPLC techniques (Chandrasekar *et al.*, 2006; Shi *et al.*, 2012; Qiu *et al.*, 2012; Yamaguchi *et al.*, 1998) and flow injection based methods

for determination of scavenging capacity against DPPH[•] were also reported (Magalhães *et al.*, 2009 d; Ukeda *et al.*, 2002; Polasek *et al.*, 2004; Koleckar *et al.*, 2008; Magalhães *et al.*, 2006a). A DPPH[•]-based optical sensor for screening of antioxidant activity was introduced by Steinberg and Milardovi'c, 2007.

In the present work, we have studied the feasibility of using a graphite ion-selective membrane sensor for evaluation of DPPH[•] scavenging activity of certain antioxidants and using BHA, a standard antioxidant, to study its response. The proposed sensor can be considered superior to the common colorimetric method since it can be used for evaluation of antioxidant activity of colored or turbid solutions and it apply neither sophisticated instruments nor any separation step, also the proposed method is more simple, faster and cheaper than amperometric and chromatographic methods.

EXPERIMENTAL

Apparatus

Mettler Toledo compact titrator model G20 with Labx software version 3.1 accompanied with Ag/AgCl double junction reference electrode was used for potential measurements. A Jenway pH meter 3310 pH/mV/ °C meter with Jenway pH glass electrode (UK) were used for pH adjustments.

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Thermostatic multiple water bath, model BT-15 (Spain). Thermometer. Double beam Shimadzu (Japan) 1601Pc UV-VIS spectrophotometer connected to a computer fitted with UVPC personal spectroscopy software version 3.7

Chemicals and reagents

2, 2-Diphenyl-1-picrylhydrazyl radical (DPPH[•]), 99.98%, was obtained from Sigma Aldrich, Cairo, Egypt. Hepamol[®] tablets (500 mg paracetamol/tablet, Hikma Pharmaceutical Company, Egypt) and Persantin tablets (75 mg dipyridamole/ tablet, Sideco Pharmaceutical Company, Egypt). All reagents and chemicals used throughout this work were of analytical grade and water used was bi-distilled. Poly (vinyl chloride) (PVC) and β -cyclodextrin (β - CD) were obtained from Fluka (Chemie GmbH, Germany), dioctyl phthalate (DOP) was obtained from Aldrich (Germany). Tetrahydrofuran (THF) was obtained from Merck (Darmstadt, Germany). Sodium hydroxide, hydrochloric acid, potassium chloride, citric acid, sodium bicarbonate, nickel chloride hexahydrate and magnesium chloride were obtained from Prolabo (Pennsylvania, USA). Simulated intestinal fluid (SIF) was prepared from 6.8 g monobasic potassium phosphate, 0.2M sodium hydroxide (NaOH) (to adjust pH to 6.8) and water to 1000 ml and the temperature was adjusted to 37 ± 0.2 °C.

Procedures

Fabrication of the membrane sensor

In petridish (5 cm diameter) 0.04 g of (β -cd) was mixed with 0.19 g PVC carboxylate and dissolved in 0.4 ml (DOP) and then mixed thoroughly with 5 ml (THF) till complete homogeneity, the solvent was slowly evaporated at room temperature until an oily concentrated mixture was obtained. The coated graphite sensor was constructed using a graphite bar 3 cm length, 3mm diameter). One end of the bar was used for connection, while the other, about 1 cm length, was dipped in the electro-active membrane mixture. The process was repeated several times until a layer of proper thickness was formed covering the terminal of the graphite bar. The sensor was left standing at room temperature to dry. The uncoated end of the graphite rod was sealed in a poly tetra ethylene tube; the tube was filled with metallic mercury into which a copper wire was dipped.

Sensor calibration

The sensor was conditioned by soaking in 10^{-2} mol L⁻¹ DPPH[•] solution for only 2 hours before measurement, storage was in distilled water when not in use, the conditioned sensor was calibrated by separately transferring 50 ml aliquots of solutions covering the concentration range of (10^{-7} to 10^{-2} mol L⁻¹) DPPH[•], into a series of 100 ml beakers. The sensor system was immersed in each solution with constant stirring at speed 20% in conjunction with Mettler Toledo reference electrode. The sensor was washed in distilled water between measurements. The sensor potential was plotted versus each negative logarithmic concentration of DPPH[•], the calibration plot obtained was used for subsequent measurements of unknown samples.

Effect of pH and temperature

The influence of pH on the response of the membrane sensor was checked at various pH values over a pH range of (1-9). 1×10^{-3} and 1×10^{-4} mol L⁻¹ DPPH[•] solutions were prepared. Effect of temperature also was checked by applying the same procedures at all concentrations at different temperatures using water bath and thermometer to adjust the temperature of each concentration.

Sensor selectivity

The potentiometric selectivity coefficients ($K_{A,B}^{pot}$) of the proposed sensor towards different substances were determined by a separate solution method using the following equation (IUPAC 2000).

$$-\log(K_{A,B}^{pot}) = (E_1 - E_2) / (2.303RT/Z_A F) + (1 - Z_A/Z_B) \log a_A$$

where $K_{A,B}^{pot}$ is the potentiometric selectivity coefficient, E_1 is the potential measured in 10^{-3} mol L⁻¹ DPPH[•] solution, E_2 is the potential measured in 10^{-3} mol L⁻¹ interferent solution, Z_A and Z_B are the charges of DPPH[•] and interfering ion, respectively, a_A is the activity of DPPH[•] and $2.303RT/Z_A F$ represents the slope of the investigated sensor (mV/concentration decade).

Potentiometric determination of % DPPH[•] scavenging of pure paracetamol and dipyridamole

50 mL of BHA, paracetamol (0.02-0.3 mg.mL⁻¹) and dipyridamole (1-4 mg.mL⁻¹) standard solutions were taken separately to obtain concentrations (10-150 μ g.mL⁻¹) for BHA and paracetamol. For dipyridamole concentrations (0.5-2 mg.mL⁻¹) are obtained, 50 ml DPPH[•] working standard solution (1×10^{-4} mol L⁻¹) were added, BHA prepared in methanol but paracetamol and dipyridamole prepared in water: methanol (1:1) then incubated at room temperature for 15 min after good shaking. Control solutions were prepared, in which no drug was added and the same procedure was carried out. The reagents solutions were prepared daily. Percentages scavenging and IC₅₀ values were calculated using an algorithm (Locatelli et al., 2009) implemented in Microsoft Visual Basic[®] 6.0 (Microsoft Corporation, Redmond, WA, USA). The software can be freely downloaded at <http://www.pharm.unipmn.it/rinaldi/software/blesq/BLLeSq.html>. Results were compared with that obtained from the colorimetric method.

Potentiometric determination of % DPPH[•] scavenging of paracetamol and dipyridamole in dosage forms

The suggested procedure was applied for the evaluation of DPPH[•] scavenging activities of dipyridamole in Persantin[®] tablets and paracetamol in Hepamol[®] tablets in which ten tablets were weighed and their mean weight was determined. The tablets were finely powdered and an accurately weighed portions of powders were transferred to 100-mL volumetric flasks, then the volume was completed with water: methanol(1:1) then filtered for each. The same previous procedure was then repeated. Percentages scavenging of paracetamol and dipyridamole in dosage forms, separately, were calculated.

Potentiometric determination of % DPPH[•] scavenging of paracetamol and dipyridamole in dosage forms in (SIF)

The same procedure was applied but the volume was completed with simulated intestinal fluid (SIF): methanol (1:1) then filtered. Percentages scavenging of paracetamol and dipyridamole in tablet dosage forms, separately, were calculated.

Colorimetric analysis

The DPPH[•] scavenging activity was measured by following the methodology described by Sharififar *et al.*, 2007 where 5 mL of BHA, paracetamol (0.02-0.3 mg.mL⁻¹) and dipyridamole (1-4 mg.mL⁻¹) standard solutions were taken separately to obtain concentrations (10-150 µg/mL) for BHA and paracetamol and (0.5-2 mg.mL⁻¹) for dipyridamole, 5 ml DPPH[•] working standard solution (1×10⁻⁴ mol L⁻¹) were added. BHA solution was prepared in methanol but paracetamol and dipyridamole solutions were prepared in water: methanol (1:1) then incubated at room temperature for 15 min after good shaking. Absorbances were measured at 517 nm. Control solutions were prepared, in which no sample was added and the same procedure was carried out. The assays were carried out in triplicate. The percent radical scavenging activity is determined from the difference in absorbance of DPPH[•] between the control and samples by the following equation:

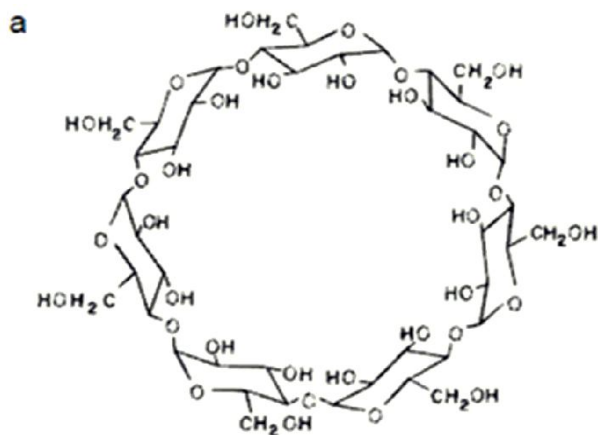
$$I\% = [(A_0 - A_1) / A_0] \times 100$$

where A₀ is the absorbance of the control reaction and A₁ is the absorbance in the presence of the antioxidant (Gu[•]lcin *et al.*, 2004). The same procedure was applied on pharmaceutical formulations and in SIF.

RESULTS AND DISCUSSION

Sensor fabrication

The central cavity of the cyclodextrin molecule is lined with skeletal carbons and ethereal oxygens of the glucose residues (fig.1a).



It has therefore lipophilic cavity into which suitably sized drug molecules are included. No covalent bonds are formed or broken during drug-cyclodextrin complex formation (Loftsson and

Brewster, 1996). We proposed that the two benzene rings of DPPH[•] are located well inside the cavity with the tri nitro benzene group protruding from β-cd cavity with the formation of hydrogen bonds between hydroxyl groups of β-cd and nitro groups of DPPH[•] (fig. 1b).

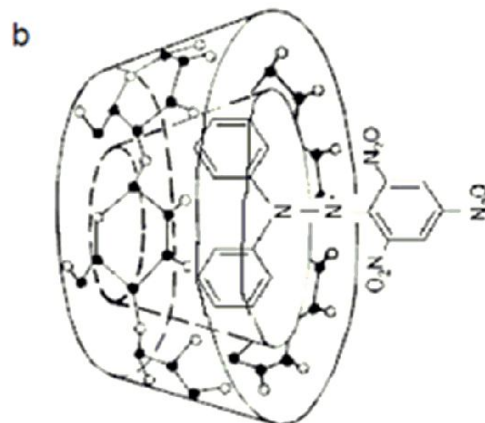


Fig. 1 (a): The chemical structure and (b): The mode of attachment between β-cyclodextrin molecule and DPPH.

It has been reported that PVC matrix is a regular support and reproducible trap for ion association complex in ion selective electrodes (Adhikari and Majumdar, 2004). Nevertheless, its use creates a need for plasticization and places a constraint on the choice of mediator (Cunningham, 1998). In the present study, DOP was used in the sensor fabrication, which plasticized the membrane and adjusted permittivity of the final organic membrane (Stefan *et al.*, 1999).

The electrochemical cell of the suggested membrane sensor for the determination of DPPH[•] can be illustrated diagrammatically as follows:

Double junction Ag/AgCl reference electrode || Test solution (DPPH[•]) | Membrane (PVC, β-CD, DOP) | Graphite rod || Metallic mercury.

Choice of solvent

The reaction mechanism between DPPH[•] and antioxidant molecule is based on an electron transfer reaction (Foti *et al.*, 2004), as a result, the scavenging capacity against DPPH[•] radical is strongly influenced by the solvent and the pH of reaction (Magalhães *et al.*, 2007b). It was concluded that 50% (v/v) aqueous/methanol solutions are a suitable choice for lipophilic and hydrophilic antioxidants and the reaction rate between DPPH[•] and the antioxidant may increase considerably with increasing water ratios. However, at water content over 60% (v/v) the measured antioxidant capacity decreased, since a part of the DPPH[•] coagulates and it is not easily accessible to the reaction with the antioxidant (Stasko *et al.*, 2007).

Sensor calibration and response time

The electrochemical performance characteristics of the investigated DPPH[•]- selective membrane sensor were evaluated

according to IUPAC recommendation data (IUPAC, 2000) and summarized in table 1.

Table 1: General Characteristics of the proposed DPPH[•]-selective membrane sensor.

Parameter	Value
Slope (mV/decade) ^a	58.50
Intercept (mV)	299.2
Correlation coefficient	0.9995
Response time(Sec.)	10
Working pH range	3-8
Concentration range (M)	10 ⁻⁶ - 10 ⁻² mol L ⁻¹
Stability (weeks)	3
Average recovery (%) ± SD ^b	99.87±0.617
LOD (M) ^c	1×10 ⁻⁶
Repeatability (SD _r) ^d	0.540
Intermediate Precision (SD _{im}) ^e	0.950
Ruggedness ^c	99.80 ± 0.840

^{a, d, e} Average of three determinations ^c Limit of detection (measured by interception of the extrapolated arms of fig.2).^b Average recovery percent of determining 10⁻⁴, 10⁻³, 10⁻² mol L⁻¹ DPPH[•] for the studied electrode using jenway pH meter 3310 pH /mV /°C meter instead of. Mettler Toledo automatic titrator.

The potential displayed by the proposed electrode for the measurements of standard drug solution in the same day and linearity range from day-to-day not vary by more than ± 1 mV. Calibration slopes didn't change by more than ± 1 mV/decade concentration over a period of 3 weeks. The required time for the sensor to reach values within ± 1 mV of the final equilibrium potential after increasing the drug concentration 10-folds was found to be 10 seconds. Typical calibration plot is shown in fig. 2. The slope of the calibration plot was 58.5 mV/concentration decades. The slight deviation from the ideal Nernstian slope (60 mV) stems from the fact that the electrode responds to the activities of drug anion and cation rather than its concentration Nernstian relation of the sensor is:

$$E = -58.5 \log [C] + 299.2$$

where [C] is the molar concentration. The detection limit of the proposed sensor was estimated according to the IUPAC definition (IUPAC, 2000). LOD value was found to be 10⁻⁶ mol L⁻¹.

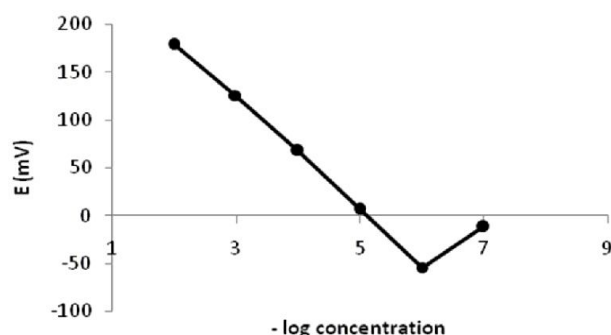


Fig. 2: Profile of the potential in mV vs. -log concentration of DPPH[•] using the investigated DPPH[•] sensor.

The effect of pH and temperature

The influence of pH on the potential response of the sensor was studied at different concentrations, 10⁻³ and 10⁻⁴ mol L⁻¹ over the pH range 1-9, the potential pH profile (fig. 3) indicated that the sensor potential is fairly constant over the pH

range of 3-8, therefore, this range can be chosen as the working pH range for the sensor assembly, above pH 8, the hydroxide anion reacts with DPPH[•], in two ways, the first one in which the anion acts as a nucleophile and makes a complex, which decomposes after that by losing a hydride anion or a nitrite anion, leading to different compounds, or the DPPH[•] is strong enough to abstract one electron from the anion and to oxidize it to the short-lived radical X, which reacts with DPPH[•], yielding also finally the nitro derivative of DPPH[•] (Ionita, 2005) as shown in fig. 4.

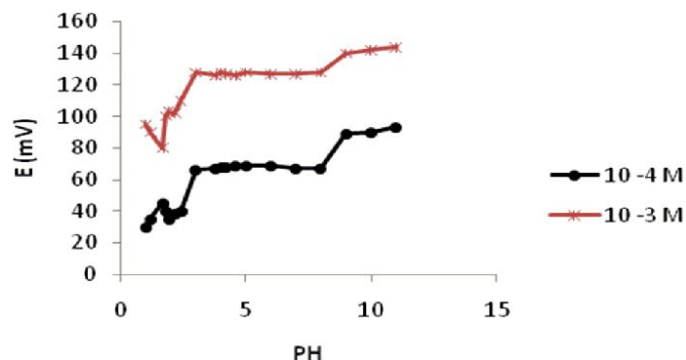


Fig. 3: Effect of pH on the response of DPPH[•] sensor.

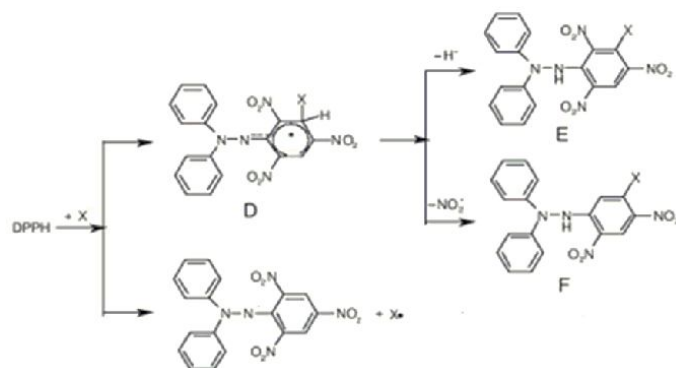


Fig. 4: The reaction of DPPH with anion (X= OH).

Upon studying the effect of temperature, the proposed sensor exhibits slight increase in its potentials as the temperature rises in the range of 25-40 °C; however, the calibration graphs obtained at different temperatures were parallel as shown in fig. 5.

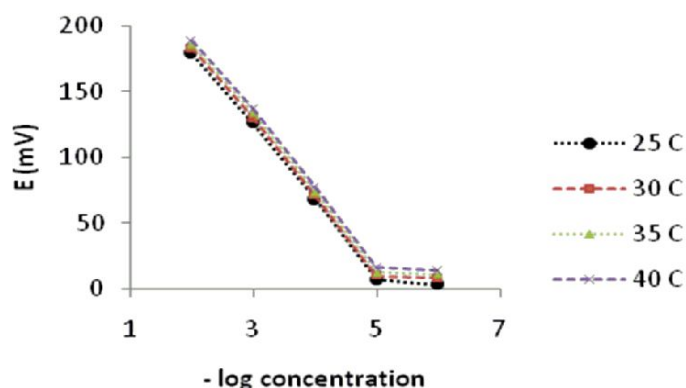


Fig. 5: Effect of temperature on the response of DPPH[•] sensor.

The limit of detection, slope and response time didn't significantly vary with variation of temperature, indicating reasonable thermal stability up to 40°C.

Sensor selectivity

Table 2 shows the potentiometric selectivity coefficients of the proposed sensor in the presence of other interfering substances. The results reveal that the proposed membrane sensor displays high selectivity.

Table 2: Potentiometric selectivity coefficients (K^{pot}) for the investigated DPPH[•] selective membrane sensor.

Interferent ^a	Selectivity coefficient
Sodium bicarbonate	1.75×10^{-3}
Citric acid	7.19×10^{-3}
Potassium chloride	1.50×10^{-3}
Nickel chloride hexahydrate	1.40×10^{-2}
Magnesium chloride	2.53×10^{-3}
Paracetamol	3.75×10^{-3}
Dipyridamole	1.33×10^{-3}
BHA	4.01×10^{-3}

^a Aqueous solutions of 1×10^{-3} M were used.

Assessment of the antioxidant activity of paracetamol and dipyridamole in pure form, dosage forms and SIF

The radical scavenging activity was calculated as follows:

$$I\% = [(E_0 - E_1) / E_0] \times 100$$

Where E_0 is the potential of the control and E_1 is the potential in the presence of the test compound at different concentrations.

The IC_{50} values were calculated graphically by plotting the antioxidant drug concentration vs. the corresponding scavenging effect using the algorithm implemented in Microsoft Visual Basic[®] 6.0 (Microsoft Corporation, Redmond, WA, USA) on the basis of probit, logit and angular regressions as presented in fig. 6-8.

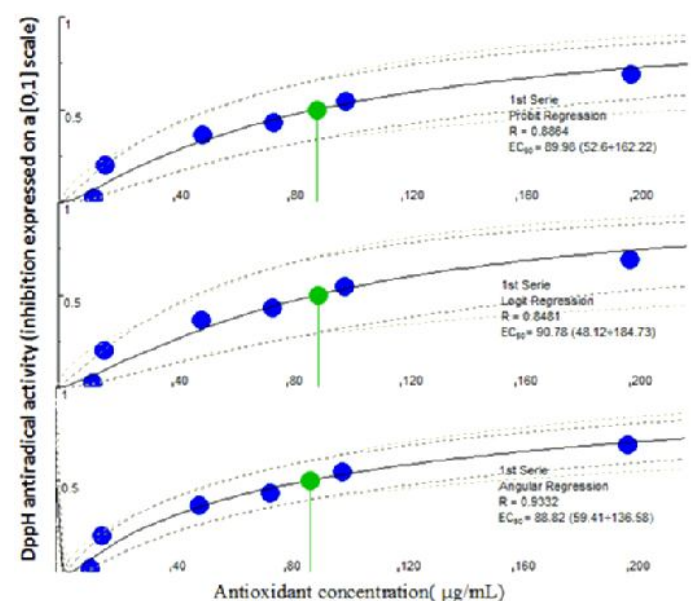


Fig. 6: Antiradical activity curves obtained for paracetamol on the basis of probit, logit and angular regressions.

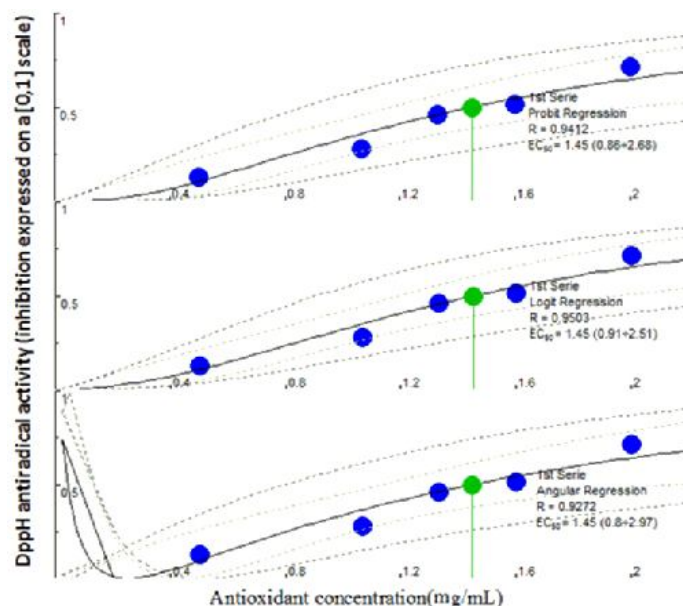


Fig. 7: Antiradical activity curves obtained for dipyridamole on the basis of probit, logit and angular regressions.

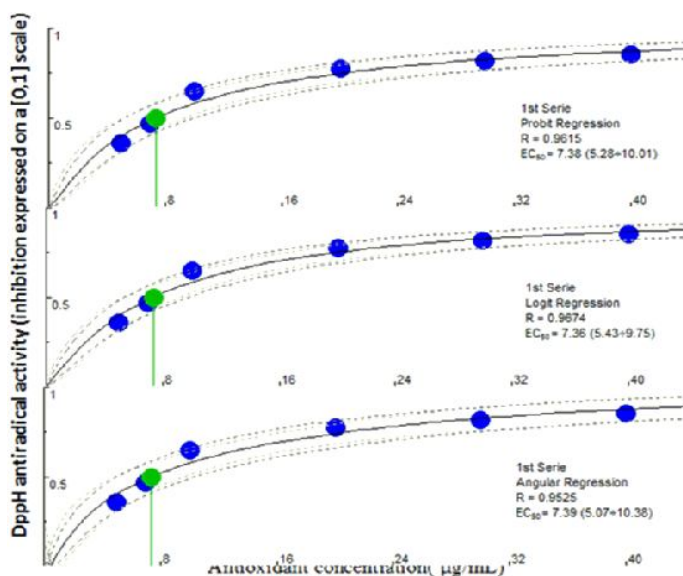


Fig. 8: Antiradical activity curves obtained for BHA on the basis of probit, logit and angular regressions.

The proposed sensor was successfully used for evaluating the antioxidant activities. The percentage scavenging of BHA ($IC_{50} = 7.38 \mu\text{g/mL}$), paracetamol ($IC_{50} = 89.98 \mu\text{g/mL}$) and dipyridamole ($IC_{50} = 1.45 \text{ mg/mL}$). Probit model had been chosen as it is well adapting to the data obtained from the colorimetric DPPH[•] assay and it generally gives the intermediate IC_{50} amongst the three regression models considered. Results arranged the scavenging activity in the following order: BHA > paracetamol > dipyridamole. The AAI value [final concentration of DPPH[•] in $\mu\text{g}\cdot\text{mL}^{-1}$ / IC_{50} ($\mu\text{g/mL}$)] of the standard antioxidant BHA is correlated well with that obtained from the application of the colorimetric method established by Gourine *et al* [6] (table 3).

Table 3: DPPH[•] radical scavenging activity of studied antioxidants

Compound	IC ₅₀ concentration ^a (µg/mL) determined by	
	Proposed sensor	Colorimetric method
Pure antioxidants		
BHA	7.38 ± 0.35	7.40 ± 0.12
Paracetamol	89.98 ± 0.45	90.05 ± 0.60
Dipyridamole	1450.00 ± 1.50	1449.00 ± 0.90
Drug formulations		
Hepamol [®]	90.05 ± 1.07	91.01 ± 0.54
Hepamol [®] in SIF	88.50 ± 0.02	89.11 ± 0.64
Persantin [®]	1451.12 ± 2.22	1450.90 ± 1.09
Persantin [®] in SIF	1449.50 ± 2.01	1452.33 ± 2.03

^aEach value is mean ± S.D. of triplicate analysis.

The results proved the applicability of the proposed sensor for the evaluation of DPPH[•] scavenging activity of the studied drugs in their pharmaceutical formulations and in SIF. Also the proposed method utilized the power of the automatic compact titrator in accurate measuring of the potential developed per second and recording of the obtained data. Also the proposed method utilized the power of the automatic compact titrator in accurate measuring of the potential developed per second and recording of the obtained data.

CONCLUSION

The proposed membrane sensor was successfully used for assessment of DPPH[•] scavenging of paracetamol and dipyridamole in pure form and in dosage form in (SIF) utilizing the power of potentiometry for the first time.

It also offers moderate stability time, elimination of drug pretreatment or separation steps, wide pH range, low detection limit and direct determination of drugs scavenging effects in turbid and colored solutions without interference by pigments or excipients. In addition, DPPH[•] radical is stable, commercially available, and does not have to be generated before assay. The use of the proposed sensor is particularly suited to fast response and low-cost screening of the antioxidant activity.

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