SYNTHESIS, ANTIOXIDANT ACTIVITY OF 2-FERROCENYLANILINE AND BIS-2 FERROCENYLANILINE AND SOME BINDING PARAMETERS TOWARDS SUPEROXIDE ANION RADICAL

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ABSTRACT

In this work, 2-ferrocenylaniline and bis-2-ferrocenylaniline were synthesized through the arylation of ferrocene by diazotasing of 2-nitroaniline by sodium nitrite in the presence of HCl to form ortho-nitrobenzendiazonium chloride as the first step, then 2-ferrocenylaniline and bis-2-ferrocenylaniline were obtained by the reduction respectively of 2-nitroppenylferrocene and bis-2-nitrophenylferrocene by $NaBH_4$ in presence of (10%) Pd/Cin methanolic medium. The synthesized compounds were characterized by IR infrared and NMR magnetic resonance spectroscopy. The superoxide scavenging effect and the phosphomolybdenum test were respectively assessed by electrochemical assay: cyclic voltammetry and by spectrophotometric assay. In this paper, the antioxidant capacity index (IC_{50}), the antioxidant activity coefficients (K_a), the interaction coefficients (K_b) and the binding free energy (ΔG) of these two ferrocene derivatives towards the superoxide radical were determined using the values of the intensities of the anodic currents from the cyclic voltammograms of the $O_2 / O_2^{\cdot-}$ couple.

KEYWORDS: *A*rylation, *R*eduction, *F*errocenylanilines, *A*ntioxidant activity, *C*yclic voltammetry.

1. INTRODUCTION

Free radicals in biological systems are ubiquitous and can come from various extracellular sources (UV radiation, X-ray and gamma radiation, atmospheric pollution, cigarettes) and intracellular (mithocondria via its respiratory chain, oxidation of small molecules: cyclooxygenases and lipooxygenases, xanthine oxidase and NADPH oxidase, endoplasmic reticulum and peroxisomes), Unbalanced diet is another cause of the formation of free radicals. The reaction of these reactive oxygenated and nitrogen species with the lipid molecules and their interactions with the nucleic acids and the proteins causes certain alterations and change

their functions**(i)**. Compounds capable of retarding or inhibiting oxidative damage to a target molecule are called antioxidants, an antioxidant molecule can react with the free radical and neutralize it by giving one of their electrons, while preventing cellular and tissue damage**(ii)**. Nitrophenylferrocenes and Ferrocenylanilines were found to have great importance in medicinal chemistry**(iii)** as antibacterial, antigungal agent**(iv)** and antioxidant**(v)**; they have obtained directly from one of the most important molecules: it is ferrocene.

The history of ferrocene begins a century ago, in 1951**(vi)**, with the temptation to synthesize fulvalene by Keally and Pauson while Miller, Tebboth and Tremain try to synthesize $Fe(C_5H_5)_2$. Then, on X-ray analysis results, in 1952, Fischer proposed a double cone structure**(vii)**. Wilkinson and Woodward proposed the structure "syndwich" on the basis of infrared data and polarity measurements**(viii)** giving rise to a new discipline: organometallic chemistry. Fischer and Wilkinson were rewarded for their pioneering work on the complexes "syndwiches" by the Nobel Prize for Chemistry in 1973**(ix)**.

Ferrocene has particular physicochemical properties, among the properties exploited for the creation of compounds with biological activities: its structural diversity, the possibility of exchange of its ligands, its catalytic and its redox properties. A diversity of ferrocenic molecules have proved their use as antitumor agents**(x-xiii)**, antiviral agents**(xiv)**, antibacterial**(xv-xvii)**, antiparasitic**(xvii, xviii)**, antifungals**(xix)** and antioxidants**(xx-xxv)**.

In this work, ferrocene-incorporated aniline compounds were successfully synthesized and characterized by various physicochemical and spectroscopic methods, studied their antioxidant capacity by spectrophotometric and electrochemical assays, and some binding parameters: the antioxidant activity coefficient (K_a), the binding coefficient (K_b), and binding free energy (ΔG) of these compounds with the superoxide anion radical.

2. MATERIALS AND METHODS

2.1. Reagents and instruments

2-nitrophenylferrocene, sodium borohydride 99% (Merck, Germany), Pd/C10% from Sigma-Aldrich, hydrochloric acid 32% (Merck, Germany), dichloromethane (Sigma-Aldrich, USA), sodium hydroxide (Merck, Germany), méthanol 99% (Biochem chemopharma), Sulfuric acid (98%), molybdate d'ammonium, ammonium phosphate (NaH_2PO_4), dimethylformamide (DMF, 99.9%) from Biochem Chemopharma (Canada), tetrabutylammonium tetrafluoroborate (Bu_4NBF_4, 99%) from Sigma-Aldrich, ascorbic acid (99%) was from Alfa Aeser used as standard antioxidant.

2.2. Instrumentation

Spectrophotometrical measurments were carried out using Spectrophotomter UV-visible (PRIM Advanced SCHOTT instruments Gmbh). Electrochemical measurements were carried using Voltalab 40 model PGZ301 potentiostat/ galvanostat in an electrochemical cell of volume V=25mL. All the measurements were made using conventional three electrode; glassy carbon (0.03 cm^2) served as the working electrode, a saturated calomel electrode (SCE) functioned as a reference and platinum wire functioned as a counter-electrode.

2.3. General procedure of synthesis

▪ **2-ferrocenylaniline (2-FA)**

2-Nitrophenylferrocene and bis-2-nitrophenylferrocene were synthesized according to the literature**(xxvi)**, it consists of a diazotization reaction of 2-nitroaniline with sodium nitrite in an acidic medium. To obtain 2-ferrocenylaniline, a suspension of 0.05 g of 10% palladium- on-

charcoal in 10 mL of water was placed in a conical flask and (0.12 g, 0.003mol) of sodium borohydride in 2.5 mL of water was added, a magnetic stirrer bar was introduced and a slow stream of nitrogen was passed through the stirred mixture. A solution of 0.5 g of 2-nitrophenylferrocene in methanol was added dropwise from a reparatory funnel during 15 min. The mixture was stirred for further 20 min then it was filtered. The filtrate was acidified with 2M hydrochloric acid to destroy excess of borohydride and then neutralized with dilute sodium hydroxide, and then, the product was extracted with dichlorométhane from the neutralized solution. Evaporation of the dried dichloromethane solution gave 2- ferrocenylaniline (0.38g, 76%) as brown oil.

IR (vcm^{-1}): 3394.5(F¸ vNH$_2$); 2962.5-2873.7(m¸ v Fc); 1627.8(F¸ vc=c); 1380.9(m¸ vC-C) (m¸ vC-C); 1029.9(F¸ v FC); 883.3(v substitution aromatic); 462.9(vFc-C$_P$). **RMN ^{1}H** (400 MHz, CDCl$_3$); δ = 7.28 CDCl$_3$; 2.38(t, 2H, NH$_2$); 4.11(s, 5H, C$_4$); 4.36(t, 2H, C$_2$ ou C$_3$); 4.48(t, 2H,C$_3$ ou C$_2$); 7.32(d, 1H, C$_9$); 7.48(t, 1H, C$_7$); 7.53(t, 1H, C$_8$); 7.82(d, 1H, C$_{10}$). **RMN ^{13}C** (400 MHz, CDCl$_3$ δ = 68 (2C, C$_2$ ou C$_3$); 69.50(2C, C$_3$ ou C$_2$) ; 70.08(5C, C$_4$) 81.45(1C, C$_1$); 123.27 (1C, C$_7$); 126.55(1C, C$_9$) 130.94 (1C, C$_5$); 132.11 (1C, C$_{10}$); 133.28(1C, C$_8$); 149.83(1C, C$_6$).

- **Bis- 2- ferrocenylaniline (bis-2-FA):**

Bis-2-FA (1, 56 g) was obtained from bis- 2-nitrophenylferrocene following the same procedure as the preparation of 2-ferrocenylaniline. Crystallization of the product from ethanol gave orange plates. Mp= 122°C. **IR (KCl disc):** 3390, 3320, 1605, 750 cm^{-1}. **^{1}H RMN (CDCl$_3$):** 4.05 (4H, s, NH$_2$), 4.25 (4H, m) and 4.48 (4H, m, C$_5$H$_4$), 6.55 (4H, m) and 7.50 ppm (4H, m C$_6$H$_4$) [26].

2.4. Antioxidant activity

2.4.1. Total antioxidant capacity by phosphomolybdenum assay

The phosphomolybdate (PPM) test was carried out according to the method described by Prieto et al(**xxvii**). It consists of introducing into tubes 100 μL of each product mixed with 0.1 ml of reagent composed of H$_2$SO$_4$ (0.6 M), NaH$_2$PO$_4$ (28 mM) and ammonium molybdate (4 mM). The tubes were then tightly closed and then incubated at 95°C for 90 minutes. After cooling, the absorbance was measured at 695 nm. The phosphomolybdenum reduction potential of the compound was expressed as equivalent of ascorbic acid (μg. L^{-1} AAE) using the following equation obtained from the standard ascorbic acid graph:

$Y = 0.003X + 0.143$ ($R^2 = 0.956$)

Y is the absorbance at 695 nm and X is the equivalent of ascorbic acid.

We plot the function AAE = f (C of compound), we find linear equations with which we calculate the IC$_{50}$ of the total antioxidant activity.

2.4.2. Superoxide scavenging test

The inhibitory activity of the superoxide anion radical is determined by cyclic voltammetry, this test is based on the Bourvellec et al method with some modifications(**xxviii**). Prior to use, the working electrode is polished, rinsed with distilled water, and dried with paper towels. This cleaning procedure is always applied before each electrochemical measurement. The superoxide radical anion is generated by commercial molecular oxygen dissolved in DMF which contains 0.02M Bu$_4$NBF$_4$ at room temperature (28 ± 1) °C. The scanning rate is maintained at 100mV/s. The applied potential range was -1.6V to 0.0V vs SCE. The studied ferrocene derivatives are added to the superoxide radical dissolved in the DMF and the voltammograms have been recorded.

- **Antioxidant activity index (IC$_{50}$%)**

The inhibition percentage of superoxide (O$_2{}^{•-}$) caused by the addition of the ferrocenic compounds is calculated by the following formula : **(xxix)**

$$Superoxide\ inhibition\ (\%) = \frac{Ip0 - Ips}{Ip0} \times 100 \quad (01)$$

Ip0, Ips is the intensities of the anodic peak current of oxygen respectively without and with test compound.

- **Antioxidant Activity coefficient**

The relative capacity of 2-FA and Bis-2-FA to scavenge superoxide radical was determined as antioxidant activity coefficient (K$_{ao}$). The constant K$_{ao}$ is defined as the ratio of current density values, with and without the addition of substrate to the free radical**(xxx)**.

$$Ka = \frac{\Delta J}{(J0 - Jres)\ \Delta C} \quad (2)$$

Where:
ΔJ: change in the oxygen anodic current density with the addition of substrate.
J0: limiting current density of oxygen without the substrate in the solution.
Jres: residual current density of oxygen
ΔC: change in the concentration of substrate (mol/L).

- **Binding constant**

The interaction strength between the superoxide radical and the antioxidant was quantified by a parameter named kb binding constant, this constant was calculated using the following equation**(xxix, xxx)**

$$Log\ \frac{1}{[AO]} = log\ Kb + log\ \frac{Ip}{Ip - Ip0} \quad (3)$$

Where:
Ipo, Ip: peak currents of superoxide anion radical in the absence and presence of additives, respectively
[AO]: concentration of antioxidant

- **Binding free enrgy (ΔG)**

The binding free energy ΔG was calculated using the following equation*(4)* **(xxxi)**.

$$\Delta G = -RTlnK \quad (4)$$

Where;
ΔG: binding free energy, KJ.mol^{-1}
R: gas constant; 8.32 J.mol^{-1}.K^{-1}
T: absolute temperature equal to 298K

3. RESULTS AND DISCUSSION

3.1. Synthesis

The synthesis of the ferrocenylanilines (2-FA, bis-2-FA) was achieved in two steps. In the first step; 2-nitrophenylferrocene and bis-2-nitrophenylferrocene (a) were made by the coupling of ferrocene with diazonium salt of 2-nitroaniline (2-nitrobenzendiazonium chloride) using phase transfer catalyst. In next step, the 2-nitrophenylferrocene and bis-2-nitrophenylferrocene were converted into corresponding ferrocenylanilines by their reduction with palladium on charcoal and sodium borohydride in methanolic medium.

2-nitrophenylferrocene is the first product recovered from the chromatographic column using a mixture of petroleum ether/ diethyl ether as eluent with a polarity gradient of 8/2 while bis-2-nitrophenylferrocene is the second product eluted from the column.

In the FT-IR spectra, peaks were observed in the range 3366 -3239 cm^{-1} those can be assigned to N-H bonds. The C=C stretching band for the test compounds were detected from 1605 to

1610 cm^{-1} and a characteristic peak for Fe-C associated with the ferrocene group was observed in the range of 447- 480 cm^{-1}.

For 2-FA, its proton NMR spectrum shows different peaks, a peak at 2.38 ppm corresponds to 2H of the amine function (NH$_2$), a peak at 4.11 ppm corresponds to the 5H of the pentadienyl ring of ferrocene (C$_5$H$_5$), a peak at 4.36 ppm corresponds at 2H linked to C$_2$ or C$_3$, a peak at 4.48 ppm corresponds to 2H linked to the C$_3$, 7.53 ppm corresponds to H of C$_8$ and 7.82 ppm corresponds to the H of C$_{10}$.

For Bis-2-FA, its proton NMR spectrum shows different peaks, a peak at 4.05 ppm corresponds to 2H of the amine fonction (NH$_2$), a multiplet at 4.25 ppm and a multiplet at 4.48 ppm corresponds to 4H of pentadienyl ring of ferrocene (C$_5$H$_4$), a multiplet at 6.55 ppm corresponds to 4H and a multiplet at 7.50 ppm corresponding to 4H of C$_6$H$_4$ ring.

3.2. Antioxidant activity

3.2.1. Total antioxidant capacity (TAC) by phosphomolybdenum

The total antioxidant capacity test is a spectroscopic method used to quantitatively determine the antioxidant capacity, by the formation of phosphomolybdenum complex. The assay is based on the reduction of Mo(III) to Mo(V) by the sample and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH. The total antioxidant capacity is calculated by the method described by Prieto and al.

Table1: Total antioxidant capacity of 2-FA and bis-2-FA expressed as ascorbic acid equivalents (mg AAE/ml).

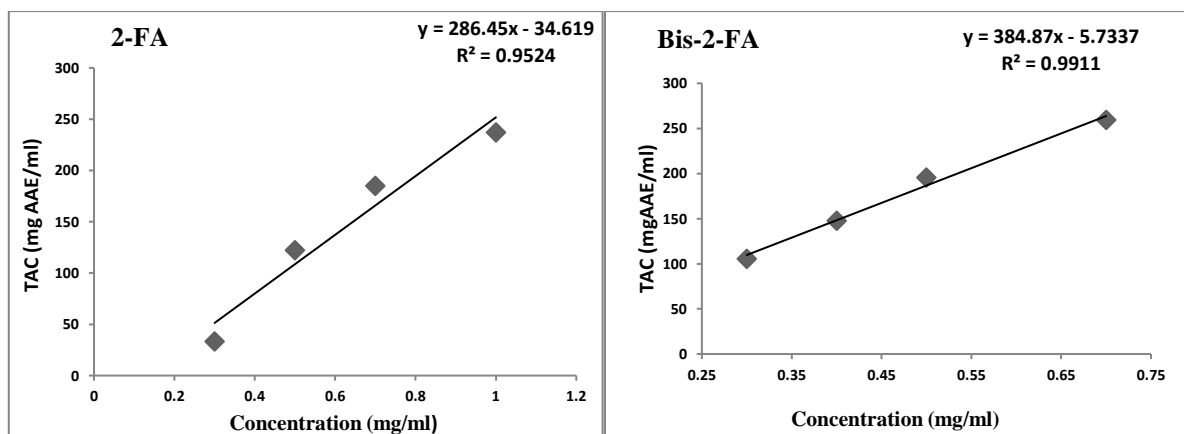| Compound	Concentration	Absorbance	TAC (mg AAE/ml)
2-FA	0.3	0.243	33.33
	0.5	0.51	122.33
	0.7	0.698	185
	1	0.854	237
Bis-2-FA	0.3	0.46	105.66
	0.4	0.586	147.66
	0.5	0.73	195.66
	0.7	0.921	259.33

Figure (1): TAC of 2-FA and bis-2-FA expressed as Equivalent Ascorbic acid

3.2.2. Superoxide scavenging test

- **Voltammetric behaviour of superoxide anion radical :**
The electrochemical techniques used to produce O$_2$$^{\cdot-}$ by reduction of dissolved oxygen in aprotic solvents are convenient processes due to the ease of the procedure; in addition no sous product is generated. The cyclic voltammetry technique is used to generate the superoxide

radical in the diffusion layer of the glassy carbon electrode by the one-electron reduction of molecular oxygen in DMF. The presence of the $O_2^{\cdot-}$ radical is easily detected by its anodic oxidation current measured at the same electrode during the return scan. The reduction of $O_2^{\cdot-}$ is a reversible reaction and it is known that the $O_2^{\cdot-}$ radical is stable in aprotic media and disproportionation does not occur during cyclic voltammetry measurements in a DMF solution. Therefore, it is a convenient way to generate $O_2^{\cdot-}$ without enzymatic systems and study its reaction with a molecule provided that the substrate is inactive in the potential range of oxygen reduction(**xxix, xxx**).
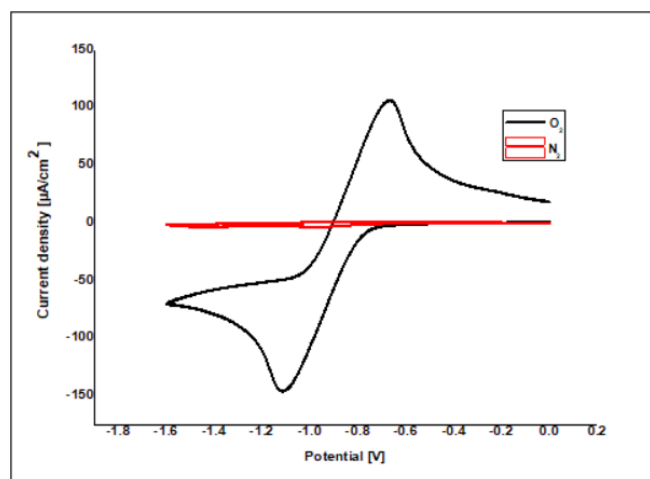

Figure (2): Cyclic voltammograms of (a) medium (DMF + 0.02 M Bu$_4$NBF$_4$) and (b) $O_2^{\cdot-}$ in the same medium on GC as working electrode vs SCE as reference at 28°C with scan rate of 100 mV/s

The cyclic voltammogram of superoxide anion radical showed one electron reversible process (figure. 2), having well developed and clear oxidation and reduction peaks with peak separation (ΔE_p) value of $66\pm3mV$, well in agreement with the reported data. The height of the voltammogram corresponds to the solubility (concentration) of the oxygen. $O_2^{\bullet-}$ is stabilized by the ion pair formation and solvation process with tetra butyl ammonium per chlorate cation. Here the oxygen radical can act as a strong Bronsted base, a nucleophile and as a one electron donor(**xxxii**)

- **Voltammetric behaviour of superoxide radical in the presence of ferrocenic derivatives**
  The cyclic voltammograms of superoxide anion radical in the presence of 2-FA and bis-2-FA are presented in Figure 2. The addition of ferrocenic compounds decreases the anodic currents from 129.76 to 121.84µA/ cm^2 for 2-FA and from 135.2 to 128.75 µA/cm^2, for bis-2-FA(Table 3), and shifts both peak positions towards more positive values. Maximum effect was observed up to 62.58. 10^{-5}M concentration while further additions of 2-FA had little effect on the peak potentials and peak currents. The decrease in the anodic current of superoxide anion radical is attributed to the presence of some antioxidants which react with superoxide radical and decrease its concentration at/around the electrode surface
  The decrease in the anodic current is attributed to the decrease of the radical concentration upon scavenging while the simultaneous decrease in the cathodic current indicates electron transfer (ET) mechanism from the antioxidant to the superoxide radical immediately followed by a proton transfer.
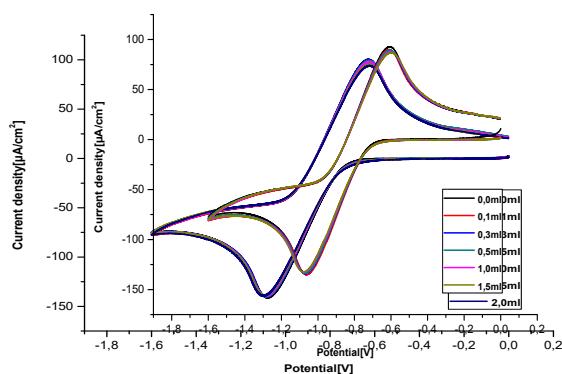
Figure (3): Cyclic voltammograms of $O_2^{\cdot\,-}$ in the presence of different concentration of 2-FA and bis-2-FA in DMF + 0.02 M Bu_4NBF_4 on GC as working electrode vs SCE at 28°C with scan rate of 0.1 V/s
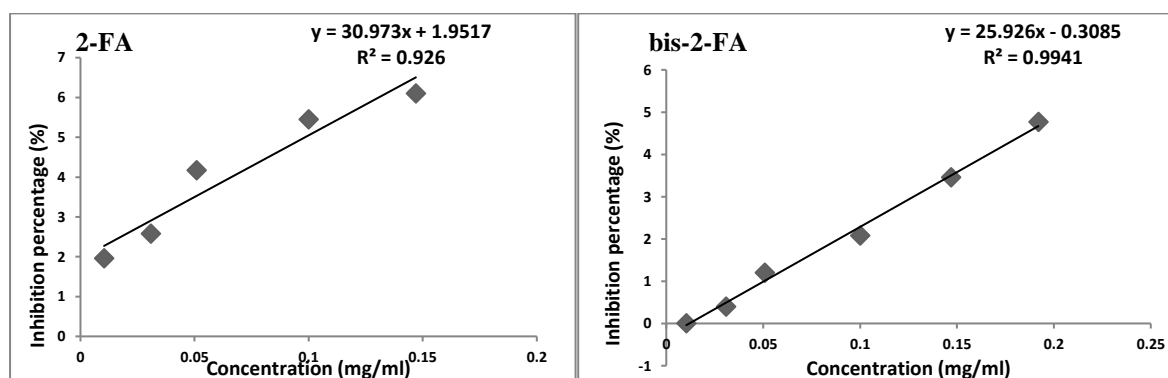

Figure (4): Plotting of scavenging of superoxide anion against the corresponding concentration of 2-FA and bis-2-FA

- **Antioxidant activity index (IC$_{50}$)**

   Applying the above equation (1), we can calculate the percentages of superoxide inhibition caused by the addition of ferrocene derivatives

   The table (4) regroup the IC_{50}, K_{a0} and K_b values; IC_{50} which is the concentration of the scavenger to cause loss of superoxide activity was calculated from the graph plotted between percentage of inhibition and concentration of the sample (for 2-FA: I (%) = 30.97C+ 1.952, $r^2 = 0.926$, for bis-2-FA: I (%) = 25.92C- 0.308, $r^2 = 0.994$).

   2-FA and bis-2-FA show a moderate antioxidant effect with IC_{50} values equal to 1.551 mg/mL and 1.940 mg/mL, respectively. We can say that the two ferrocenic compounds are bad scavengers of superoxide radical ion.

   From the cyclic voltammograms, we can deduce the anodic current densities of 2-FA and bis-2-FA witch illustrated in table (2)

Table (2): anodic current densities of 2-FA, bis-2-FA at different concentrations

C.10^5 (M)	Ip ($\mu A/cm^2$)		Log(1/[AO])		Log Ip/(Ip0-Ip)	
	2-FA	**Bis-2-FA**	**2-FA**	**Bis-2-FA**	**2-FA**	**Bis-2-FA**
0	129.76	135.2	/	/	/	/
3.35	127.21	135.19	4.47	4.47	1.697	4.13
10.03	126.41	134.66	4.00	4.00	1.576	2.39
16.59	124.35	133.58	3.78	3.78	1.361	1.92
32.59	122.68	132.38	3.48	3.48	1.24	1.671

47.91	121.84	130.51	3.31	3.31	1.187	1.45
62.58	/	128.75	/	3.20	/	1.300

- **Binding constant (K_b)**

The interaction strength between the superoxide radical and the antioxidant was quantified by a parameter named kb binding constant.

The figure (5) presents Plots to determine binding constant (k_b) using equation log (1/[AO]) vs log [Ip/(Ipo – Ip)] for 2-FA and bis-2-FA.
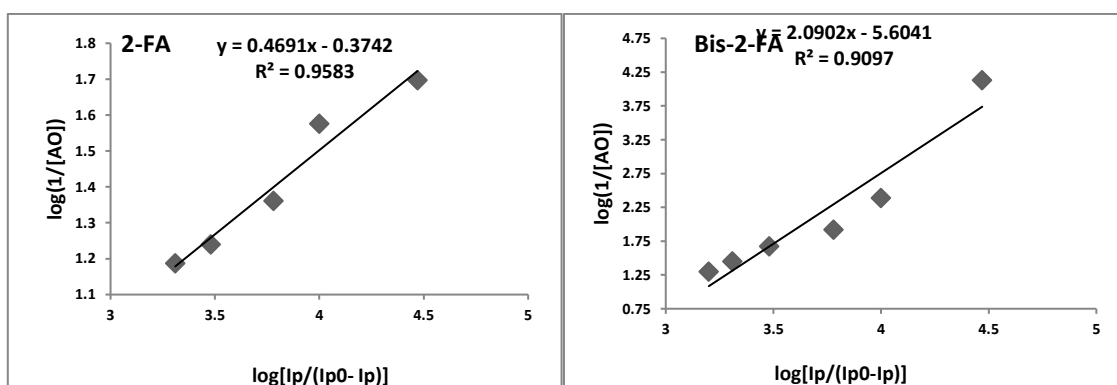

Figure (5): Plots to determine binding constant (k_b) using equation log (1/[AO]) vs log [Ip/(Ipo – Ip)] for 2-FA and bis-2-FA.

The additive which gives high value of binding constant shows strong interaction with the radical. From Table 2 it is evident that the percentage decrease of the anodic current of superoxide anion radical and binding constant (K_b) values the following order:

2-FA < bis-2-FA

- **Antioxidant Activity coefficient (K_a)**

The relative capacity of 2-FA and Bis-2-FA to scavenge the target radical was determined as antioxidant activity coefficient (K_a). The constant K_a is defined as the ratio of current density values, with and without the addition of substrate to the free radical.

Table (3): parameters deduced to calculate K_{ao}

$C\ 10^5$ (M)	J ($\mu A/cm^2$)		$J/J0$- Jres	
	2-FA	**Bis-2-FA**	**2-FA**	**Bis-2-FA**
0	129.76	135.2	/	/
3.35	127.21	135.19	0.98	1.00
10.03	126.41	134.66	0.97	0.996
16.59	124.35	133.58	0.96	0.988
32.59	122.68	132.38	0.94	0.979
47.91	121.84	130.51	0.939	0.96
62.58	/	128.75	/	0.95

The figure (6) presents the relative change of the $O_2^{\bullet-}$ current density vs. change in sample concentration for anodic peak of 2-FA and bis-2-FA.
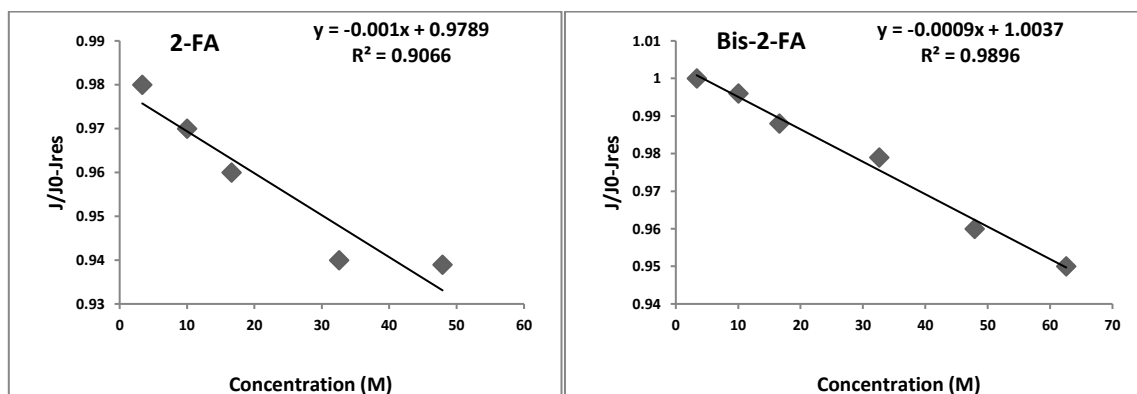
Figure (6): Relative change of the $O_2{}^{\bullet-}$ current density vs. change in sample concentration for anodic peak of 2-FA and bis-2-FA.

- **Binding free energy (ΔG)**

The negative values of free energy support that the binding of 2-FA and bis-2-FA to superoxide is spontaneous and exothermic(**xxxiii**). The ΔG values in table 4 indicates more affinity of superoxide to 2-FA followed by bis-2-FA which is consistent with the above mentioned results(**xxxiv**).

The results: IC_{50}, K_a, K_b and ΔG are reported in the Table 4.

Table (4): IC_{50}, binding parameters (K_a, K_b and ΔG) of 2-FA and bis-2-FA with superoxide anion radical

compound	IC_{50} (mg/ml)	K_a (M^{-1})	K_b	ΔG (KJ.mol^{-1})
2-FA	1.551	0.001	2.94	-2.261×10^3
Bis-2-FA	1.940	0.0009	123.083	$- 12.035 \times 10^3$

4. CONCLUSION

The current work was aimed to synthesize two ferrocene derivatives, 2-ferrocenylaniline and Bis- 2-ferrocenylaniline and investigate their antioxidant character against superoxide anion radical while employing cyclic voltammetry method and their total antioxidant capacity by phosphomolybdenum test. The outcome of the work can be summarized into: All the two compounds cause a little decrease in the anodic current of superoxide anion radical which evidently demonstrates their potential antioxidant activity. Quantification, in terms of binding constant (K_b), antioxidant activity coefficient (K_a) and binding free energy (ΔG) fully complements the radical scavenging ability of the ferrocenic compounds.

5. ACKNOWLEDGEMENTS

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Received on April 17, 2021.