ESR, ELECTROCHEMICAL AND ORAC STUDIES OF NITRO COMPOUNDS WITH POTENTIAL ANTIPROTOZOAL ACTIVITY

MARÍA ARAVENA C. [1,] *ROBERTO FIGUEROA* [1], *CLAUDIO OLEA-AZAR.* [1]*, *VICENTE J. ARÁN* [2]

[1)] *Departamento de Química Inorgánica y Analítica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Olivos 1007, Independencia, Santiago, Chile (caolea@ciq.uchile.cl).*
[2)] *Instituto de Química Medica, Consejo Superior de Investigaciones Científicas, Madrid, España*
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ABSTRACT

Cyclic Voltammetry (CV) and electron spin resonance (ESR) techniques were used in the investigation of several potentially antiprotozoal nitro derivatives of dihydroquinoxaline and indazole. A self-protonation process involving protonation of the nitro group due to the presence of an acidic proton in the structure of the nitro compound was observed in the first step of an ECErev reduction mechanism. ESR spectra of the free radicals obtained by electrochemical reduction *in situ* were characterized and analyzed. The ESR spectra showed three different hyperfine patterns for both families of compounds. In order to evaluate the free radical scavenger properties of these nitrocompounds we carried out ORAC-Fl (Oxygen Radical Antioxidant Capacity) assay. These derivatives showed a low antioxidant capacity.

Keywords: ESR; Chagas' disease; ORAC; Cyclic Voltammetry, Nitro compounds.

INTRODUCTION

The trypanosomiases form is a widespread group of parasitic diseases that affect over several hundreds of millions of people, especially in the tropical and subtropical countries [1]. The parasite *Trypanosoma cruzi* is the causative agent of Chagas' disease, an endemic infection that affects human populations mainly in Central and South America, where 18 million people are infected, and causes approximately 400,000 deaths per year[2]. Nifurtimox (Nfx) and Benznidazole (Bnz), the drugs used in the treatment of this pathology, are effective only during the acute phase of the disease. Moreover, these compounds cause serious side effects, such as peripheral neuropathy, anorexia, vomiting, allergy, and cardiac and renal toxicities[3].

Experiments carried out on the drugs used in Chagas' treatment suggest that intracellular reduction followed by redox cycling yielding reactive oxygen species (ROS) may be their major mode of action against *T. cruzi*. These ROS can cause cellular damage directly by reacting with various biological macromolecules or indirectly by generation of the highly reactive hydroxyl radical via iron-mediated Haber-Weiss and Fenton reactions[4].

On the other hand, an important number of pharmacologically active compounds including Nfx and Bnz have a nitro aromatic group in their molecular structures [5,6,7,8,9,10]. Due to the ability of these drugs to be reduced at the nitro group, they are metabolized to the corresponding amines via the formation of nitroso and hydroxylamine derivatives.

Nevertheless, it is known that the biological activity of nitro compounds is not due to the final products of reduction but to the formation of intermediates, possibly free radicals[11].

Olea-Azar C. et al. studied [12] the electrochemistry of 3-alkoxy and 3-hydroxy-5-nitroindazole derivatives by Cyclic Voltammetry, in DMSO as solvent. They found that the reduction mechanism depends on the acidic moieties in their structures. A self-protonation process involving protonation of the nitro group was observed. Some derivatives presented a one-electron reversible transfer corresponding to the generation of the nitro anion radical by an Erev mechanism. Others presented a reduction mechanism involving a self-protonation process and the generation of the nitro anion radical from uncharged species by an Erev and ECErev mechanism.

A series of indazole *N*-oxide derivatives were synthesized by Gerpe A. et al [13] and their antichagasic properties were studied. These authors found that 3-cyano-2-(4-iodophenyl)-2*H*-indazole *N*-oxide exhibited an interesting activity against the two *T. cruzi* strains and the two life-stages evaluated, and also showed leishmanicidal activity in the three parasite strains evaluated.

Rodriguez J. et al [14] studied two new groups of 5-nitroindazole derivatives using electrochemical and spectroscopic techniques. They observed a self-protonation process involving protonation of the nitro group. The reactivity of the nitro-anion radical of these derivatives with glutathione, a biologically relevant thiol, was also studied by cyclic voltammetry.

These studies demonstrated that glutathione could react with radical species containing the 5-nitroindazole system. It was also demonstrated that these nitro-anion radicals show three different patterns of delocalization in which the indazole side-chain at N1 does not have a major influence.

Another important group of nitro compounds of biological interest are the nitrofuran derivatives that have been studied in terms of their mechanisms of action and their ability to act as alternative chemotherapeutic agents for Chagas' disease[15,16,17,18,19].

Recently, we studied the effect of labile hydrogen present in the different structures of Ru and Pd complexes with nitrofuran derivates as ligands, in order to verify the ORAC-FI values, and we related these results to the biological activity of these compounds. [20,21].

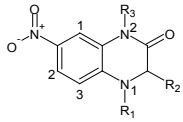
In accordance with the references discussed above, we set out to study two new families of heterocyclic derivatives of nitroquinoxaline (NQ) and 5-nitroimidazole (NI) with the aim of evaluating their physical-chemical properties in order to characterize them as new potential antichagasic drugs.

The study of these two families (Tables 1 and 2) is mainly based on the scaffold and the functional groups of these molecules. These compounds have a nitro group (RNO_2) that could be reduced to generate the respective anion radical ($R\text{-}NO_2^{\cdot-}$) and, in some cases, an amide (H-N-CO), amine ($R\text{-}NH_2$) or other group bearing a labile hydrogen (thiosemicarbazones and semicarbazones) could easily give up a hydrogen atom [12,13,14,20,21,22,23]. This ability could quench the free radical generated and thus modify their antiprotozoal capacity.

The electrochemical behavior of these derivatives was studied in DMSO using cyclic voltammetry, and the radical species were characterized using electron spin resonance.

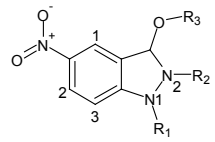
In discussing the electrochemical assays, these will be separated into two sections; one for molecules without labile hydrogen and another for those that possess labile hydrogen in their structure.

Table 1: *Chemical structure of Dihydroquinoxaline derivatives.*

	R_1	R_2	R_3
NQ-1	$-(CH_2)_6-Cl$	-H	-H
NQ-2	$-CH_3$	-H	$-CH_3$
NQ-3	$-(CH_2)_6-Cl$	-H	$-CH_3$
NQ-4	$-(CH_2)_3-$		-H
NQ-5	$-(CH_2)_5-$		-H
NQ-6	$-CH_2-Ph-CH_2-$		-H

e-mail: colea@uchile.cl

Table 2: *Chemical structure of 5-Nitroindazole derivatives.*

	R_1	R_2	R_3
NI-1	-H	-H	-(CH_2-*O*-Cl-Ph)
NI-2	-(CH_2)$_5$-		---
NI-3	-(CH_2-Ph)	-CH_3	---
NI-4	-CH_3	-(CH_2-Ph)	---

EXPERIMENTAL

Samples

The NQ and NI (Tables 1 and 2) were synthesized according to reported methods [14,24,25a,b].

Cyclic voltammetry

Dimethylsulfoxide (DMSO) was obtained from Aldrich. Tetrabutylammonium perchlorate (TBAP), used as a support electrolyte, was obtained from Fluka. CV was carried out using a Metrohm 693 VA instrument with a 694 VA Stand convertor and a 693 VA Processor, in DMSO (ca. 1.0 x 10^{-3} mol/L), under a nitrogen atmosphere at room temperature, with TBAP (ca. 0.1 mol/L), using a three-electrode cell. A hanging mercury drop electrode was used as the working electrode, a platinum wire as the auxiliary electrode, and saturated calomel as the reference electrode.

ESR spectroscopy

ESR spectra were recorded in the X band (9.85 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 KHz field modulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G. The anion radicals were generated by electrolytic reduction *in situ* under the same conditions of temperature, atmosphere and concentrations stated for the CV experiment. The ESR spectra were simulated using the program WINEPR Simfonia version 1.25.

Oxygen Radical Absorbance Capacity Assay (ORAC-Fluorescein)

Chemicals.

2,2'-Azo-bis(2-amidinopropane) dihydrochloride (AAPH) was used as the peroxyl radical source. Fluorescein (FL) and Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid), were purchased from Sigma-Aldrich.

Solutions and ORAC determination.

The methodology proposed by Ou et al. [26] was modified for this study. The reaction involving AAPH, fluorescein (FL) and antioxidant (NI and NQ) was carried out in 3000 µL quartz cells. Constant quantities of AAPH and FL were added to the cell while the volume of phosphate buffer (75 mM, pH 7.4) was decreased to compensate the volume of the compounds studied. For all measurements 240 mL 12 mM AAPH, and 215 mL of 70 nM FL, were added [21].

The five nitro compounds studied (NQ-1,4,5,6, NI-1) were dissolved in DMSO to obtain a concentration of 10^{-4} M. Increasing amounts ranging from 15 to 60 mL were added, with final concentrations between 0.5 and 2.0 mM.

The standard reference compound Trolox used to build the calibration curve was dissolved in phosphate buffer (75 mM) in a range of concentrations from 0.5 to 2.0 mM.

A blank without antioxidant was prepared under the same conditions as the samples, and the measurements were carried out at 50°C.

A luminescence spectrometer (Perkin-Elmer model LS 50B, Boston, MA, USA) connected to a thermostated bath (Haake Fisons model DC1-B3, Karlsruhe, Germany) was used in this analysis. To detect the fluorescence, the excitation wavelength was adjusted to 490 nm and emission was measured at 515 nm.

RESULTS AND DISCUSSION

Cyclic voltammetry

Molecules without labile hydrogen (NQ-2,3 and NI-2,3,4).

In order to achieve the best experimental conditions that ensure nitro-anion radical stability, DMSO was used as an aprotic medium and TBAP was used as the support electrolyte. Under these conditions, all compounds displayed similar electrochemical behaviour. Figure 1 shows the cyclic voltammogram of NQ-3, with only one well-defined couple with a peak EcII/EaII near -1.26 V for NQ. These derivatives reveal a quasireversible monoelectronic transfer corresponding to the generation of the nitro anion radical ($RNO_2^{\bullet-}$).

The characterization of the voltammetric wave took place using parameters DE and Ipa/Ipc. The Ipa/Ipc values were obtained from Nicholson and Shain's equation[27].

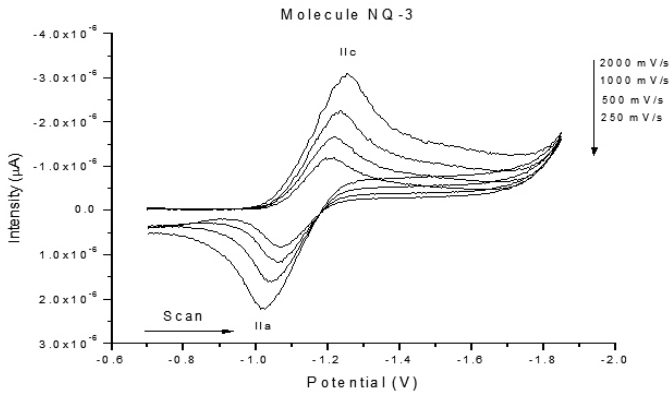
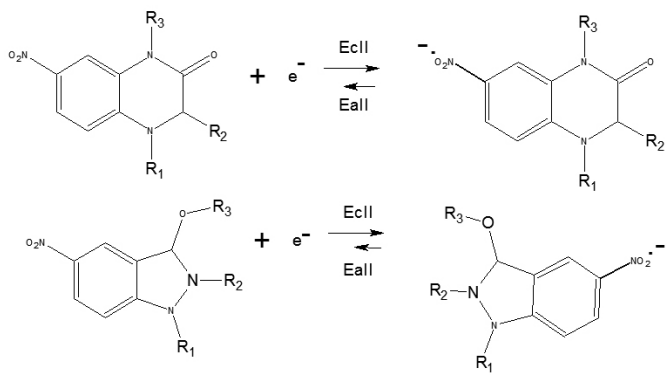


Figure 1: *Cyclic voltammogram of NQ-3 without hydrogen label in 100% DMSO with 0.1 M TBAP. Sweep rate 250 mV/s-2000 mV/s.*

The electrochemical parameters obtained for these compounds showed that when the scan rate was increased (from 0.25 to 2 V/s) the Ipa/Ipc values approached unity. The parameter DE did not reach 0.06 V for either NI or NQ derivatives, indicating that this is a quasireversible process.

For each of the compounds studied, I_{PC} vs. $v^{1/2}$ curve is linear, indicating that the electron transfers are diffusion-controlled and are not due to the species adsorbed on the surface of the electrode[28].

The reduction mechanism proposed for these compounds is shown in scheme 1.



Scheme 1: Reduction mechanism proposed for NQ and NI derivatives without labile hydrogen.

Moolecules with labile hydrogen (NQ-1,4,5,6 and NI-1)

Figure 2 shows the cyclic voltammogram of NQ-5. The electrochemical behavior is similar for these five derivatives. The voltammograms obtained for each compound showed two reduction waves, one cathodic peak EIc/EIa (c.a. −1.16 V) corresponding to nitro anion radical RNO2•− and a second wave at higher cathodic potential (EIIc/EIIa, c.a. −1.38 V), v/s SCE, corresponding to the reduction of the anion –$ORNO_2$ generated through a self-protonation reaction as a chemical complementary equilibrium (C1).

This process corresponds to an acid–base equilibrium in the aprotic medium, a typical self-protonation phenomenon displayed by nitro-compounds with acidic moieties in their structures [29,30,31].

It is possible that the higher negative potential (wave EIIc/EIIa) of the nitro derivatives corresponds to a diminished capacity to accept electrons due to its negative charge.

In order to obtain suitable conditions to observe the nitro anion radical of these derivatives and verify the self-protonation mechanism proposed, we

carried out experiments in the presence of increasing amounts of NaOH (0.1 M).

Figure 3 show the voltammogram obtained for NQ-5 in the presence of different amounts of alkali. The electroreduction wave EIc gradually disappears with increasing NaOH concentration on going from 0 to 1 mM (Fig. 3).

The calculated Ipa/Ipc ratio using the Nicholson and Shain equation [25,26] increases to about 1.0 with the addition of NaOH for peak EIIc/EIIa. These results confirm the ECErev mechanism proposed for these derivatives given by the increment in the Ipa/Ipc ratio toward the reversibility of its final peak. Figure 2 shows voltammograms characteristic of these derivatives.

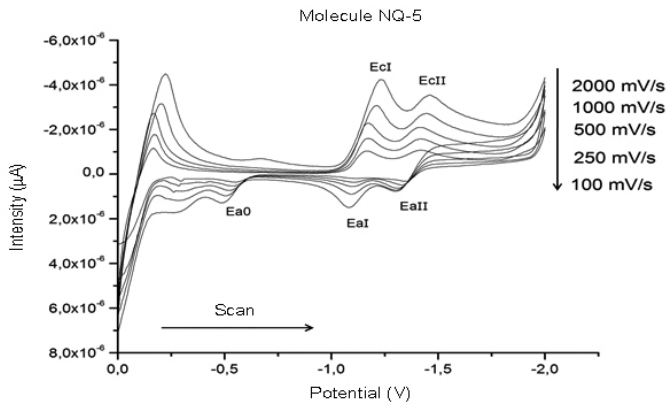


Figure 2: Cyclic voltammograms of NQ-5 with hydrogen label in 100% DMSO with 0.1 M TBAP. Sweep rate 100 mV/s-2000 mV/s.

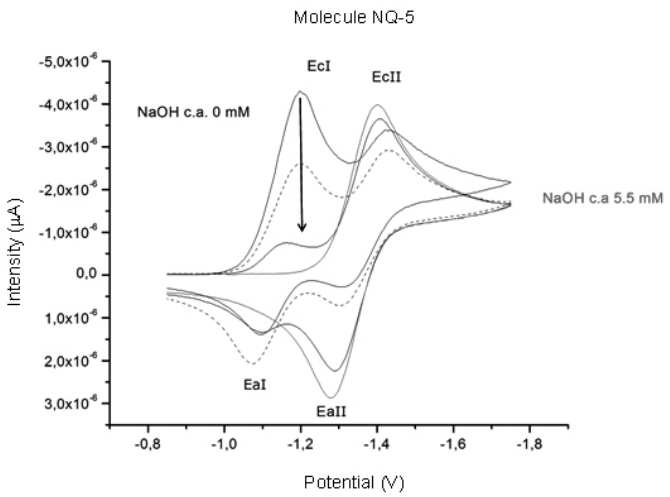
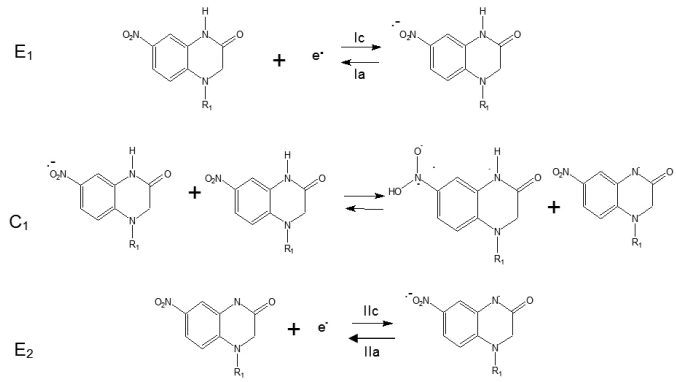


Figure 3: Cyclic voltammogram of 1x10^{-3}M NQ-5 with labile hydrogen in the presence of different amounts of NaOH (0.1M), sweep rate 2000 mV/s.

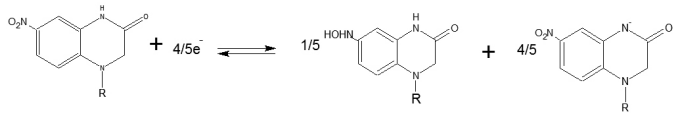
This study confirms the presence of an acidic proton in the chemical structures of NI and NQ derivatives. The proposed mechanism for the reduction of these molecules is presented in Scheme 2.



Scheme 2: Reduction mechanism proposed for NQ derivatives with labile hydrogen.

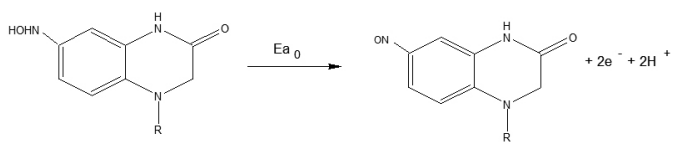
NI derivatives presented a similar mechanism to that suggested in Scheme 2 for NQ derivatives.

In addition to the two electrochemical reduction waves (E_1 and E_2), an electrochemical oxidation peak (Ea0) with a potential close to -0.5V is observed. This wave corresponds to the electrochemical oxidation of a hydroxylamine (RNHOH) to nitroso compound as described as a general mechanism of reduction of aromatic nitro compounds[32].The hydroxylamine is generated as a reduction product of the nitro compound studied as shown in Scheme 3.



Scheme 3: *Electrochemical reduction of nitro derivatives.*

With this feature it is possible to attribute the Ea0 signal to an irreversible oxidation process of the hydroxylamine to the corresponding nitro compound as shown in scheme 4.



Scheme 4: *Electrochemical oxidation of hydroxylamine to nitro compound*

The results obtained by cyclic voltammetry for all the compounds studied are summarized in Table 3.

Table 3: Cyclic voltammetric parameters in DMSO corresponding to cathodic peaks vs. saturated calomel electrode (sweep rate 2000 mV/s)*.

Structure	EcI (V)	EcII $RNO_2^{\cdot-}$ (V)	EcII $RNO_2^{\cdot-}$ (V)
NQ-1	-1.32	…	-1.54
NQ-2	…	-1.25	…
NQ-3	…	-1.26	…
NQ-4	-1.12	…	-1.23
NQ-5	-1.23	…	-1.46
NQ-6	-1.23	…	-1.44
NI-1	-1.07		-1.36
NI-2		-0.73	
NI-3		-1.09	
NI-4		-1.07	

*Concentration 10^{-3} mol/L for the nitro compounds, PTBA 0.1 mol/L, solvent DMSO, sweep rate 2000 mV/s.

Oxygen Radical Absorbance Capacity Assay (ORAC-Fluorescein)

In order to study the hydrogen donating ability of the nitro compounds and its possible relationship to trypanosomicidal activity, the free-radical scavenger capacity of NI-1 and NQ-1,4,5 and 6 was further evaluated using the ORAC assay. The $ORAC_{FL}$ assay expresses antioxidant activity relative to a standard (Trolox) while measuring the oxidation of the fluorescent substrate by peroxy radicals generated during the reaction. This method follows a hydrogen atom transfer pathway, where the antioxidant and a peroxyl radical interact with each other thus breaking the reaction of oxidation chain.

To compare the antioxidant capacity of these molecules we used Trolox (range of concentration from 0.5 mM to 2.0 mM) as a reference antioxidant (figure 4).

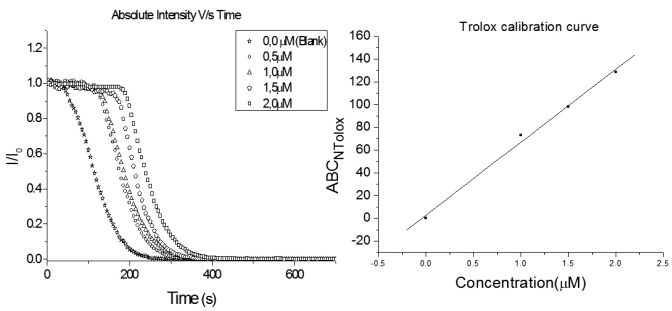


Figure 4: A) Fluorescence decay curves of fluorescein induced by AAPH in the presence of Trolox at pH 7.4 in phosphate buffer. B) Calibration curve for Trolox.

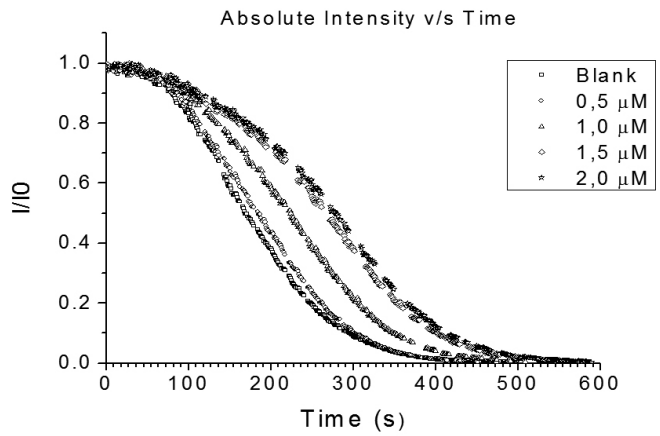


Figure 5: Fluorescence decay curves of fluorescein induced by AAPH in the presence of nitro compound NQ-1, at pH 7.4 in phosphate buffer.

Figure 5 shows the decay characteristics in the $ORAC_{Fl}$ test for nitro compounds NI and NQ.

The decay curve shown in Figure 5 is similar for the five nitro compounds studied. Here, it is possible to see a slight increase in the area under the curve from the blank with increasing concentrations of NI and NQ, which translates into a low antioxidant capacity of the compounds, to slightly retarded oxidation of fluorescein.

$ORAC_{FL}$ values of the nitro compounds studied were close to the reference value of Trolox.The compounds studied here have low antioxidant capacity. This feature indicates that the labile hydrogen present in the structure of the compound should not interfere with their ability to generate free radicals.

The results obtained by the $ORAC_{FL}$ assay for NI and NQ derivatives are summarized in Table 4.

Table 4: ORAC values of the family of nitro compounds.

Structure	$ORAC_{FL}$	R^2
TROLOX	1	0.99
NQ-1	0.69	0.93
NQ-2	--	--
NQ-3	--	--
NQ-4	1.29	0.87
NQ-5	1.39	0.90
NQ-6	0.67	0.95
NI-1	1.08	0.92
NI-2	--	--
NI-3	--	--
NI-4	--	--

Electron Spin Resonance

In order to evaluate the generation of nitro anion radical species proposed in the electrochemical mechanism, ESR experiments for selected nitrocompounds were done. NI and NQ free radicals were prepared *in situ* by electrochemical reduction in DMSO applying the potential corresponding to peak EcI or EcII obtained from the CV experiments. All the studied structures produced stable paramagnetic intermediates in the first reduction step. The interpretation of the ESR by means of a simulation process confirmed the stabilities of these radical species due to delocalized unpaired electrons. The simulated spectrum of the NQ-2 radical (Fig. 6) shows two triplets, one for the nitro group nitrogen and the other for nitrogen atom (N1) of the quinoxaline group, and four doublets for the ring H-1, H-2, H-3 and H-4.

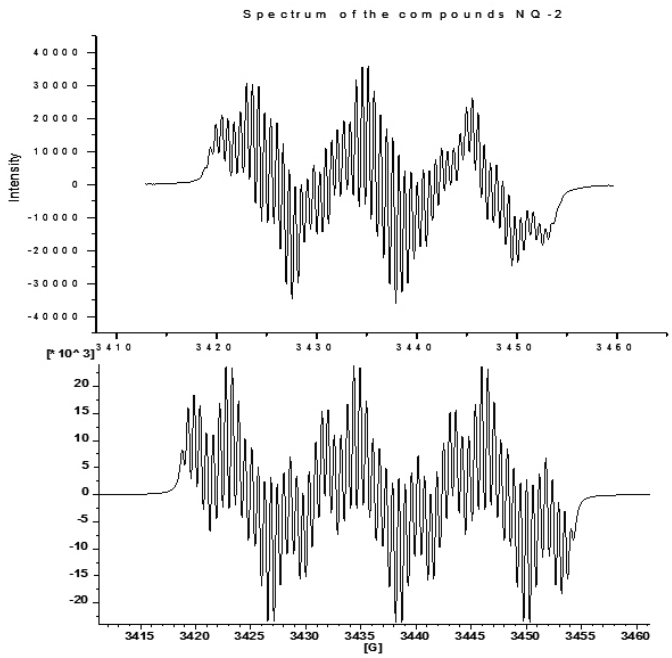


Figure 6: ESR experimental spectrum of the anion radical of NQ-2 in DMSO and computer simulation of the same spectrum. Spectrometer conditions: microwave frequency, 9.68 GHz; microwave power, 20 mW; modulation amplitude 0.20 G; receiver gain, 30 db.

The spectrum was simulated using the following parameters: line width = 0.30 G, Lorentzian/Gaussian ratio = 1 and hyperfine constants are included in Table 5.

Figure 7 displays a simulated spectrum for NI-1 in terms of three triplets corresponding to the nitrogen atoms of the nitro group and N1 and N2 of the indazole system and three doublets assigned to nuclei H-1, H-2 and H-3 of the ring.

Spectrum of NI-1

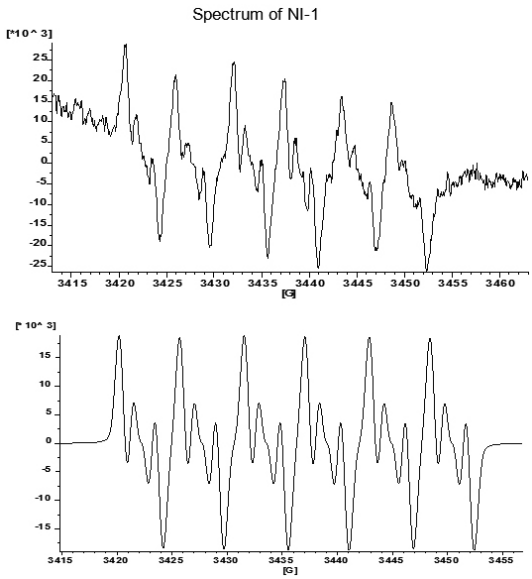


Figure 7: ESR experimental spectrum of the anion radical of NI-1 in DMSO and computer simulation of the same spectrum. Spectrometer conditions: microwave frequency, 9.63 GHz; microwave power, 20 mW; modulation amplitude 0.98 G receiver gain, 30 db. The spectrum was simulated using the following parameters: line width = 0.45 G, Lorentzian/Gaussian ratio = 0.60.

The NQ-6 free radical (Figure 8) displays a simulated spectrum of two triplets, one for the nitro group nitrogen and the other for nitrogen atom (N1) of the quinoxaline system, and three doublets due to H-1, H-2, H-3 and H-4 of the ring.

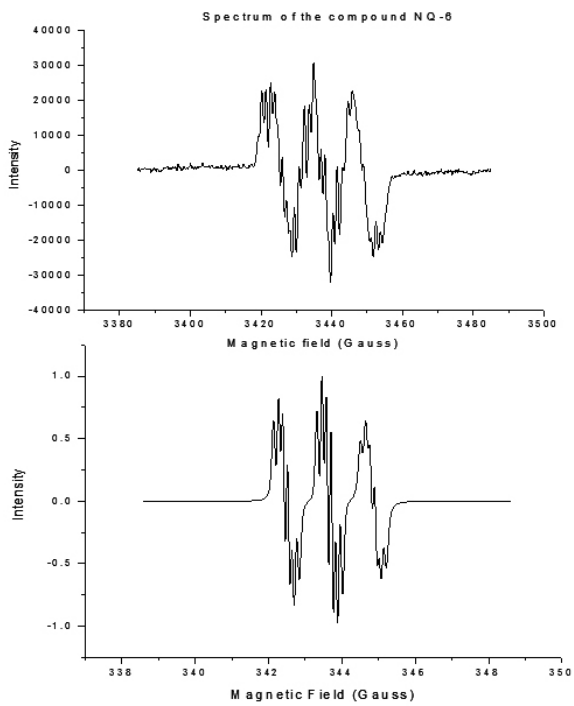


Figure 8: ESR experimental spectrum of the anion radical of NQ-6 in DMSO and computer simulation of the same spectrum. Spectrometer conditions: microwave frequency, 9.70 GHz; microwave power, 20 mW; modulation amplitude 0.98 G; receiver gain, 30 db. The spectrum was simulated using the following parameters: line width = 0.33G, Lorentzian/ Gaussian ratio = 0.5.

The three simulated spectra indicated in this report represent the hyperfine pattern for the ten nitro compounds studied.

Table 5: Hyperfine coupling constants and *g* value of the simulated NI and NQ free radical spectra (number of atoms see Table 1 and 2).

Compounds	NNO_2	N1	N2	H-1	H-2	H-3	H-4	g
NQ-6	11.95	1.2		2,35	1.37	0.37	0.3	2.017
NQ-2	11.600	1.200		3.600	3.400	1.000	0.537	2.013
NI-1	11.350	0.215	0.215	5.500	2.000	1.100		2.003

CONCLUSION

Our CV results show that NI or NQ derivatives are electrochemically reduced *via* formation of a nitro anion radical ($RNO_2^{\bullet-}$). The reduction mechanism depends on the acidic moieties in their structures. A self-protonation process involving protonation of the nitro group was also observed. The reduction mechanism proposed for derivatives with labile hydrogen is an ECErev one corresponding to the generation of the nitro anion radical from the uncharged species, followed by a self-protonation process from a hydroxyl moiety and the generation of a nitro anion radical from the negatively charged species.

On the other hand, derivatives without labile hydrogen displayed a reduction mechanism *via* formation of the nitro anion radical ($RNO_2^{\bullet-}$).

The $ORAC_{FL}$ assay showed that all compounds studied have low antioxidant capacities, indicating that the labile hydrogen present in the structure of the compounds should not interfere with their ability to generate $RNO_2^{\bullet-}$ free radicals.

Stable free radicals were generated using electrochemical reduction at potentials corresponding to the EIc and EIIc wave and were characterized by ESR spectroscopy.

The NI and NQ compounds studied showed three different spectral patterns depending on their structural characteristics. These results indicated that the side chain does not have a major influence on the electron delocalization.

The ESR spectral pattern was similar for NQ derivatives. In the case of NI compounds, the different ESR pattern with respect to the other NQ derivatives could be explained in terms of the molecular structure due to the fact that they show a structure which facilitates delocalization of the unpaired electron in the heterocyclic system.

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