Applications of Electron Spin Resonance and Spin Trapping in Tropical Parasitic Diseases

C. Olea-Azar*[1], C. Rigol[1], F. Mendizábal[2] and R. Briones[1]

[1]*Departamento de Química Inorgánica y Analítica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile*

[2]*Departamento de Química, Facultad de Ciencias Universidad de Chile*

Abstract: Free radicals may be reaction intermediates in biological systems in more situations than are presently recognized. However, progress in detecting such species by Electron Spin Resonance (ESR) has been relatively slow. ESR is a very sensitive technique for free radical detection and characterization. It can be used to investigate very low concentrations of radicals provided that they are stable enough for their presence to be detected. For unstable radicals special techniques have to be employed. One of these methods is called Spin Trapping.

Parasitic diseases in tropical and subtropical areas constitute a major health and economic problem. The range of antiparasitic drugs varies widely in structural complexity and action at the subcellular and molecular levels. However, a number of these drugs are thought to exert their action by generating free radicals. Most of the free radical producing drugs used against parasites are: quinones, naphtoquinones, quinone-imines, aminoquinolines, *N*-oxides and nitroheterocyclic compounds.

This review summarizes some of the more relevant achievements of ESR and Spin Trapping applications in parasitic diseases studies. The use of ESR spectroscopy to obtain relevant information about free radical characterization and the analysis of the mechanisms of action of drugs involved in several parasitic diseases is also presented.

Keywords: ESR, spin, trapping, free radical, parasitic diseases.

1. INTRODUCTION

Parasitic diseases in tropical and subtropical areas constitute an economic problem. Considering the progress of mankind at the end of the twentieth century, parasitic diseases are synonymous with ignorance and low level of education and income. For example Chagas' disease is endemic in Latin America, affecting 16-18 million people, with more than 100 million exposed to the risk of infection (WHO 1997) [1].

The last three decades research into free radicals has led to a better understanding of physiological and drug induced production of free radicals in biological systems, including the parasites'. The drugs used for treatment in parasitic diseases are known to act through several mechanisms. However the most studied drugs, and the ones with apparently better antiparasitic results are those which involve a free radical generation mechanism. The families studied as free radical producing drugs include quinones, quinone-imines, aminoquinolines and nitroheterocyclic compounds.

The progress in detecting such species by Electron Spin Resonance (ESR) has been relatively slow. ESR spectroscopy is a technique that allows the detection and quantification of paramagnetic species i.e. compounds with unpaired electrons. It can be used to investigate very low concentrations of radicals provided that they are stable enough for their presence to be detected [2]. In biological systems the direct detection of free radicals is often not possible because of their high reactivity and transient nature. At this time, Spin Trapping is used as a technique to make this feasible [3,4].

This review summarizes some of the most relevant achievements of interest about ESR and Spin Trapping applications in the study of tropical parasitic diseases. We also present the use of ESR spectroscopy to obtain relevant information about the mechanism of action of drugs in some parasitic diseases.

2. ESR AND SPIN TRAPPING GENERAL PRINCIPLES

2.1 Electron Spin Resonance (ESR)

Paramagnetism arises as a consequence of the presence of unpaired electrons within an atom or a molecule. It can be said that Electron Spin Resonance (ESR), often called Electron Paramagnetic Resonance (EPR), is the most direct and sensitive technique to investigate paramagnetic materials.

ESR is similar to Nuclear Magnetic Resonance (NMR), the fundamental difference being that ESR is concerned with the magnetically induced splitting of electronic spin states, while NMR describes the splitting of nuclear spin states. In both ESR and NMR, the sample material is immersed in a strong static magnetic field and exposed to an orthogonal low-amplitude high-frequency field. ESR usually requires microwave-frequency radiation (GHz), while NMR is

*Address correspondence to this author at the Departamento de Química Inorgánica y Analítica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile; Tel: +56-26782834; Fax: +56-2-7370567; E-mail: colea@uchile.cl

observed at lower radio frequencies (MHz). In ESR spectroscopy, energy is absorbed by the sample when the frequency of the radiation is coincident with the energy difference between two electronic states in the sample, but only if the transition satisfies the appropriate selection rules. Splitting can occur only when the electron is in a state with non-zero total angular momentum, i.e. electrons in atoms with closed atomic shells cannot show this behavior [2].

For a free electron the spin angular momentum can have two possible orientations and these give rise to two spin states of opposite polarity. In the absence of an external magnetic field the two spin states are degenerate. However, if an external magnetic field is applied the degeneracy is lifted, resulting in two states of different energy. This splitting is called the Zeeman effect. A peak in the absorption will occur when the magnetic field "tunes" the two spin states so that their energy difference matches the energy of the radiation.

Knowledge of the g values and the detailed hyperfine interactions (a values) allow to identify radical species, and these parameters contain information about the electron distribution within the molecule. Radicals are often present as intermediates during a reaction; consequently their identification will give information concerning the reaction mechanism and measurement of how their concentration changes with time will give kinetic data.

2.2 Spin Trapping

ESR spin trapping techniques have successfully been applied to determine and identify free radical intermediates in biology. Spin trapping allows one to determine if short-lived free radicals are involved as reaction intermediates by scavenging the reactive radical to produce more stable radicals, detectable by ESR.

The technique of spin trapping was developed in the late 1960s to facilitate the detection of reactive free radicals by ESR spectroscopy [5-8]. This method involves the addition of the spin trap, typically an organic nitrone or nitroso compound (Fig. **1**), to the radical generating system in a concentration sufficient to ensure rapid reaction with any radicals present to give stable, detectable, nitroxide radical adducts. Nitroso spin traps, of which 2-methyl-2-nitrosopropane (MNP) and 3,5-dibromo-4-nitrosobenzene sulfonic acid (DBNBS) (Fig. **2**) are the most commonly employed, have the advantage that the reactive radical attaches directly to the nitroso nitrogen atom, and is therefore in close proximity to the unpaired electron which is located primarily on the nitroxide function. This usually results in the detection of additional distinctive hyperfine couplings from magnetic nuclei present in the added radical. The size and nature of these couplings make adduct identification easier and more definitive. A number of compilations of data for such adducts are available; see also the spin trap database at the NIEHS website[1]. The relatively small amount of kinetic data available on the rates of trapping of radicals is also consistent with the rate of addition of radicals to nitroso traps being more rapid than with nitrones [4,9]. These traps do, however, have the disadvantage that they form long-living readily detectable adducts with a more limited range of radicals (usually limited to carbon-centered species) than nitrone traps. The nitroso compounds give an adduct in which the radical added is bonded directly to the nitroxide nitrogen, so that the hyperfine splittings in the ESR signal are more diagnostic of the original radical. However the adducts are less stable than nitrones' and may decay within minutes.

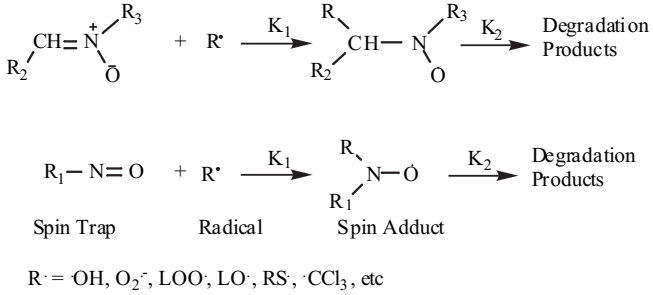

Fig. (1). Mechanism of the addition of free radical with of a spin trap typically an organic nitrone or nitroso compounds.

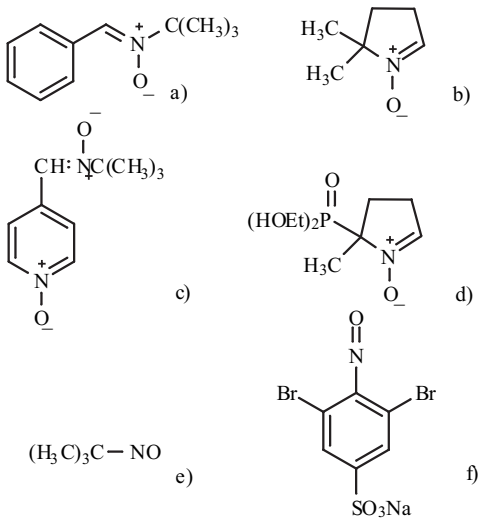

Fig. (2). Nitroso and nitrone spin traps molecular structure, a) PBN; b) DMPO; c) POBN; d) DEMPO; e) MNP; f) DBNBS.

In contrast, nitrone spin traps, including 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), *N*-tert-butyl-α phenylnitrone (PBN), and α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN) (Fig. **2**), often form long lived adducts with a wider range of radical species (e.g., carbon-, oxygen-, sulfur-, and nitrogen-centered species). However, with nitrone spin traps the reactive radical adds to the carbon atom adjacent to the incipient nitroxide group, and is therefore more distant from the molecular orbital containing the unpaired electron. As a result it is often not possible to resolve any hyperfine couplings from the added radical itself. This makes the definitive assignment of the observed spectral lines to a particular species much more complicated. However, the magnitude of the hyperfine couplings arising from the spin trap-derived nitroxide nitrogen and especially the β-hydrogen, are dependent, particularly with the cyclic nitrones, on the nature and structure of the added radical as a result of the influence of this species on the conformation of

[1] (http://EPR.niehs.nih.gov)

the nitrone. The size of these couplings can therefore provide valuable information on the nature of the radical trapped [10]. The use of spin traps to detect radicals in biological systems has been reviewed extensively [4,7].

3. BIOLOGICAL APPLICATIONS OF ESR AND SPIN TRAPPING TECHNIQUE

As introduced previously a number of antiparasitic agents have been shown to exert their actions through a free radical metabolism: nitro and *N*-oxide compounds used against trypanosomatids, anaerobic protozoa and helminths; the antimalarials primaquine, chloroquinine, and quinghaosu; and quinones active *in vitro* and *in vivo* against different parasites [11].

Nifurtimox (Nfx) and benznidazole ((**5a,5b**), (Fig. **3**) are currently used to treat Chagas' disease. In general, the biological effects of nitroheterocyclic compounds, especially in *T. cruzi*, are believe to involve redox cycling of the compounds and oxygen radical production, two processes in which the nitroanion radicals play an essential role [12-15]. A characteristic ESR signal corresponding to the nitro radical appears when Nfx is added to intact *Trypanosoma cruzi* cells, the causative agent of Chagas' disease (American trypanomiasis) [16]. This and other experiments [17] suggest that intracellular reduction of Nfx followed by redox cycling yielding $O_2^{\cdot -}$ and H_2O_2, may be the major mode of action against *T. cruzi*. However, the use of these drugs has disadvantageous side effects like fever, muscle weakness, abdominal or stomach pain, vomiting, etc [18], frequently forcing the treatment to be stopped. The mechanisms of these side-effects were not fully understood, particularly in the case of Nfx. Most studies available on the toxicology of Nfx correlate the occurrence of Nfx-induced deleterious effects with the nitroreductive biotransformation of this nitroheterocyclic compound. However, the hypothesis was advanced that peroxynitrite formation from Nfx resulting from the interaction of nitric oxide and superoxide generated during biotransformation of the Nfx might play a role in Nfx toxicity [19].

Derivatives (**1**) and (**2**) for instance (Fig. **3**) showed interesting *in vitro* trypanocidal activity but failed to be good trypanocidal agents *in vivo* because of their inherent toxicity (mostly (**2**)) [13]. The undesirable toxicity of (**1**) and (**2**) on the host is probably due to the nitro moiety which acts as a nonselective redox-damaging function.

Olea-Azar *et al*. also reported a series of nitrocompounds that generated nitro anion radicals, which was proved by ESR spectroscopy [20,21]. Recently, Olea-Azar *et al*. [22] studied new analogues of Nfx ((**3**), (**4**) (Fig. **3**). The free radical ESR spectra of these compounds is shown in (Fig. **4**). Compound (**4**) showed better or at least similar biological activity against *T. cruzi* than Nfx, and it produced oxygen redox cycling in *T. cruzi* epimastigotes. The ESR

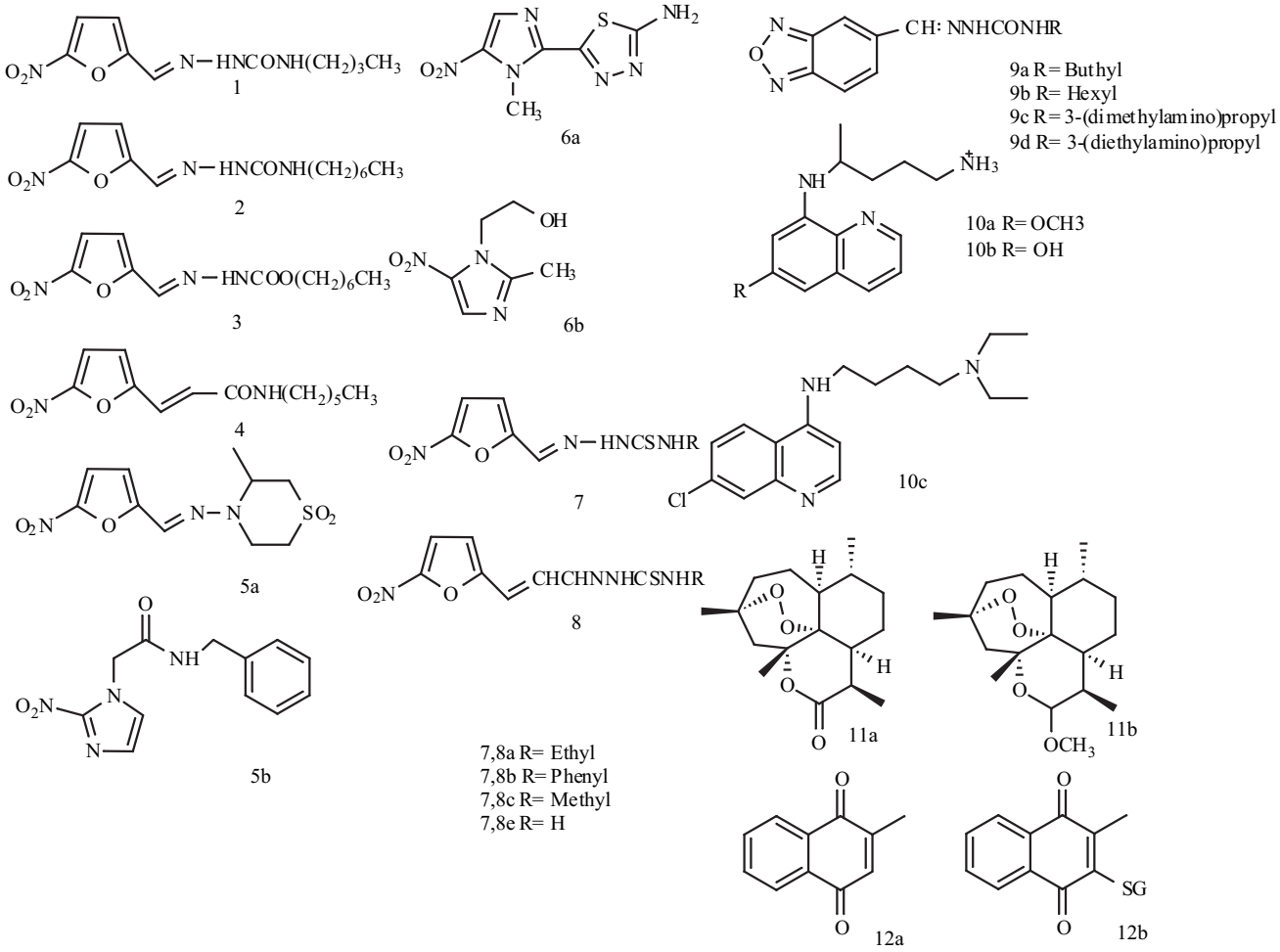

Fig. (3). Molecular structures of various antiparasitic compounds.

signal intensities were consistent with the trapping of both hydroxyl radical ((*) (Fig. **5**) and the nitrofurane ((#) (Fig. **5**) derivative radical by DMPO. These results were in agreement with the observation of increasing the oxygen uptake caused by the presence of the compound (**4**) in a *T. cruzi* incubation solution, meaning that the anti-Chagas activity of this compound was achieved by an oxidative stress mechanism.

Tsuhako *et al*. [23] studied the bioreductive activation of nitroimidazole derivative megazol (compound (**6a**), (Fig. **3**) promoted by ferrodoxin: NADP+ oxidoreductase, rat liver microsome and cellular fractions of. *T. cruzi*. Direct ESR detection and characterization by computer simulation of megazol anion radical were possible in the presence of NADPH and ferrodoxin: NADP+ oxidoreductase under anaerobic conditions. However, the megazol anion radical was not detected in the presence of either rat liver microsomes or cellular fractions of *T. cruzi*. These results indicate a restricted bioreductive metabolism of megazol and suggested that the trypanocidal activity is unrelated to a redox cycling process.

Viode *et al*. reported that megazol is a highly active compound used against several strains of *T. cruzi*. With the aim of determining the probable mode of action against the parasite, the interaction of megazol with different redox enzymes was studied and compared with that of Nfx and metronidazole (compound (**6b**), (Fig. **3**). The three nitroaromatic compounds are reduced by L-lactate cytochrome c-reductase, adrenodoxin reductase, and NADPH:cytochrome P-450 reductase, the efficiencies of the enzymatic reductions being roughly related to the reduction potentials of these pseudo-substrates. As the enzyme responsible for the reduction of megazol within the parasite has not yet been identified, the nitroimidazole was assayed with *T. cruzi* lipoamide dehydrogenase and trypanothione reductase. Megazol did not inhibit the physiological

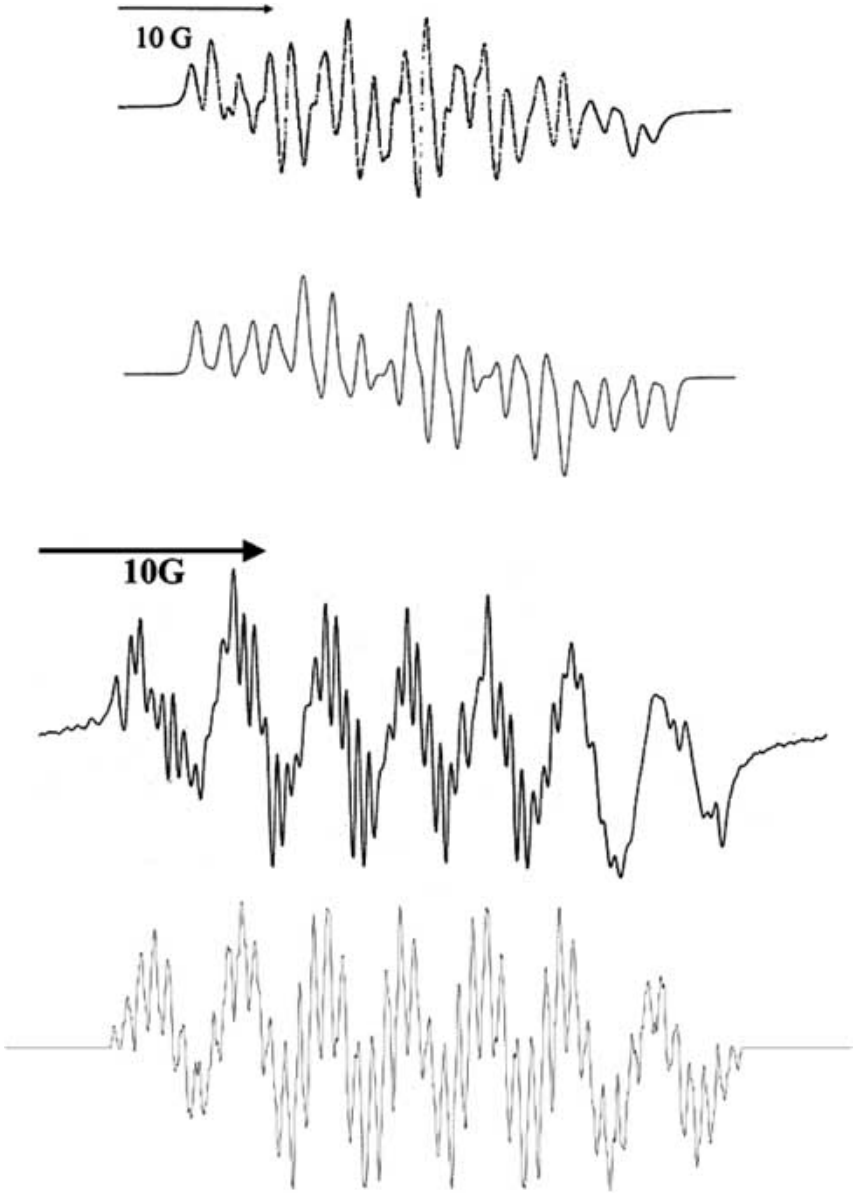

Fig. (4). (A) ESR experimental spectrum of the radical-anion of (**3**) (Fig. (**4**)) in DMSO and computer simulation of the same spectrum. (B) ESR experimental spectrum of the radical-anion of (**4**) in DMSO and computer simulation of the same spectrum.

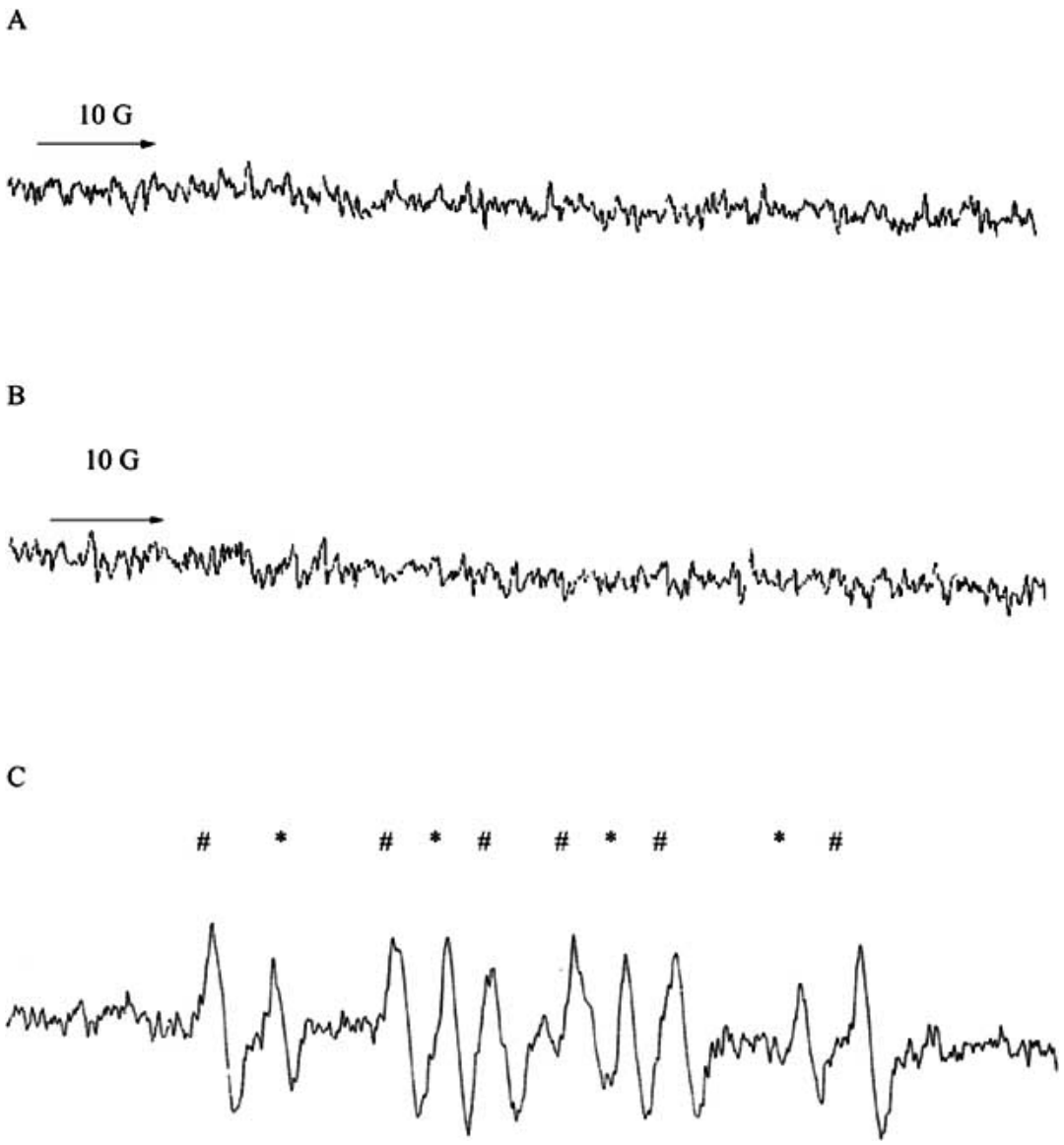

Fig. (5). ESR spectra of DMPO-OH· and DMPO-compound (**4**) (Fig. (**4**)) radical adducts obtained with *T. cruzi* extracts. The ESR spectra were observed 10 min after incubation at 37°C with *T. cruzi* microsomal fraction (4 mg protein/mL), NADPH (1mM), EDTA (1mM), in phophate buffer (20mM), pH 7,4 and (A) acetonitrile (10 v/v) and DMPO (100mM), (B) Nitro 2 (1mM in acetonitrile 10 v/v) and (C) Nitro 2 (1mM in acetonitrile 10 v/v) and DMPO (100mM) (DMPO-OH adduct (*): a_N=a_H = 14.78 G; DMPO-Nitro 2 adduct (#): a_N= 15.21 G, a_H= 23.48 G. Spectrometer conditions: microwave frequency 9.68 GHz microwave power 20 mW, modulation amplitude 0.4G, scan rate 0.83 G/s , time constant 0.25 s number scans: 10.

reactions but proved to be a weak substrate of both flavoenzymes. The single electron reduction of this compound, as well as of Nfx and metronidazole, by NADPH:cytochrome P-450 reductase, by rat liver as well as by trypanosome microsomes was confirmed by ESR experiments. The interest relies in the fact that megazol interferes with the oxygen metabolism of the parasite as Nfx and metronidazole, but its extra activity when compared to Nfx may be related to other features not yet identified [24]. These results are in agreement with Tsuhako's in terms of the fact that it seems likely that because of its high biological activity against *T. cruzi,* megazol acts on more than one single target. This acquires particular interest taking into account the severe side effects of nitrocompounds and their carcinogenicity.

Aguirre *et al*., [25] studied the *in vitro* activity and the mechanism of action against *T. cruzi* of 5-nitrofuryl containing thiosemicarbazones ((**7**), (**8**) (Fig. **3**). Free radical production was detected when the compounds were incubated in presence of mammalian liver microsomes. All the 5-nitrofuryl thiosemicarbazone derivatives were capable to produce free radicals in biological medium. So, the microsomal incubations of all the compounds gave an ESR spectrum after a brief induction period of 1–2min. The authors analyzed theoretically the biological behaviour of the studied compounds. All derivatives showed similar values of atomic charge on NO_2 nitrogen. This fact confirmed the results obtained in the ESR experiments. The compounds possess similar electrochemical behaviour, so they could act biologically in an initial redox pathway.

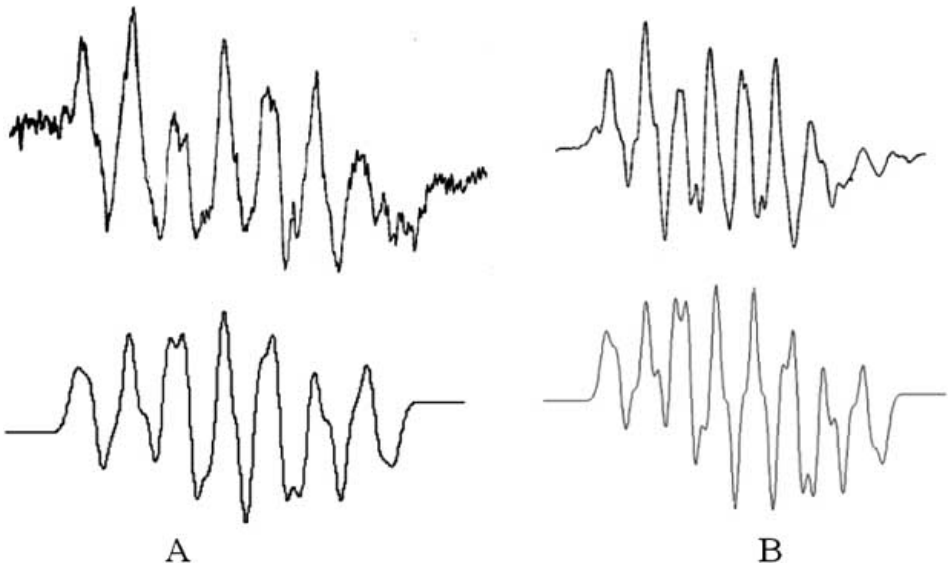

Fig. (6). A Up: ESR spectrum of (**9a**) (Fig. (**4**)) *N*-oxide derivate generated by microsomal system. Bottom: Computer simulation of the same spectrum

B Up: ESR spectrum of (**9c**) (Fig. **4**) *N*-oxide derivate generated by microsomal system. Bottom: Computer simulation of the same spectrum.

Like the nitro pharmacophore of antitrypanosomal drugs, the *N*-oxide moiety has proved to be responsible for the biological activity of different families of drugs (with antitumor or antibacterial activities) through the production of free radical species [26,27].

Cerecetto *et al*. reported studies on the 1,2,5-oxadiazole *N*-oxide family (compound (**9**), (Fig. **3**) in order to determine their antitrypanosomal activities, tested *in vitro* against the epimastigote form of *T. cruzi* [28]. Moreover, they have shown ESR spectra that prove the facile electronation of the *N*-oxide moiety. Besides, these new structures were based on the conjunction of *N*-oxide systems and the semicarbazide moieties similar to Trypanothione, substrate of Trypanothione reductase involved in the defense mechanism of trypanosomatids, against oxidative stress. Olea-Azar *et al*. characterized the free radical species of *N*-oxide families generated by microsomal reduction, using ESR spectroscopy (Fig. **6**) [29]. The hyperfine splitting pattern of these biochemically generated free radicals was the same as that obtained by electrochemical reduction. Also, the ESR spectra proved that the reduction mechanism of these compounds involves the protonation of the *N*-oxide group, as suggested by the cyclic voltammetric results.

As we can see, oxidative stress might play a key role in many fatal endpoints caused by other diseases and —at the same time— it represents a most promising rationale for e.g. antimalarial chemotherapy. The detoxification of reactive oxygen species (ROS) is a challenge for erythrocytes infected with *Plasmodia*. In this regard it is interesting to note that a number of drugs currently in clinical use exert their activities, at least in part, by increasing oxidative stress in the parasitized erythrocyte [30]. That is the case of primaquine ((**10a**) (Fig. **3**), an 8-aminoquinoline, which is the only tissue schizontocide currently available for free radical treatment of malarial infections. Its utility is compromised by its toxic effects on erythrocytes, and indeed primaquine was one of the first agents recognized to produce oxidative stress [31,32]. Despite its importance, years ago it was not clear whether the pharmacological effects of primaquine were due to the parent compound or to its metabolites. In 1988, Ohara *et al*. [33] detected, by ESR spectroscopy, during enzymatic oxidation of primaquine, a drug-derived radical. The results showed the generation of a radical species during to the oxidation of primaquine catalyzed by horseradish peroxidase-H202 or methemoglobin-H202. A complex product distribution is expected during aromatic amine oxidations, as the initial products are more easily oxidized than the parent compound, undergoing rearrangements and addition reactions. However, comparison of the obtained ESR parameters with those reported in the literature indicated that a benzidine-like rearrangement is the most plausible to happen. The further generation of a benzidine-like radical in the presence of nucleophilic groups can lead to further condensation reactions accounting for the polymeric nature of the reaction products. A similar spectrum was detected during enzymatic oxidation of 6-hydroxyprimaquine ((**10b**) (Fig. **3**) at pH 9.0. Simulations of ESR spectra indicated that the free radicals contain two primaquine moieties and the authors stated that this *in vitro* oxidation of primaquine to a free radical intermediate stable in the presence of oxygen might be considered as a new mechanistic route for analyzing the pharmacological effects of primaquine.

Various mechanisms of action have been found for the different drugs used in antimalarial chemotherapy. For example, chloroquine ((**10c**) (Fig. **3**) acts by preventing toxic haem (ferri/ferroprotoporphyrin IX, FP) detoxification and its activity can be enhanced by depletion of GSH. In this work the authors observed that the redox cycling of the metabolites of primaquine [34] (Fig. **8**) exerts a substantial oxidative stress, also artemisinin ((**11a**), (Fig. **3**) commonly known as quinghaosu is thought to react with haem moieties forming cytotoxic radicals [35].

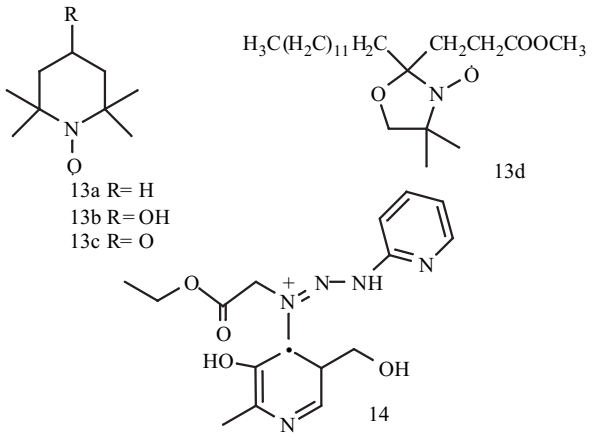

Fig. (7). Molecular structure of spin probes and iron chelator L2-9.

Deslauriers *et al*. [36] showed another mechanism of action concerning primaquine and studied it using ESR. Erythrocytes from normal mice and mice infected with the malarial parasite *Plasmodium berghei* reduce the water-soluble spin probes, stable paramagnetic compounds, usually nitroxides, 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO (**13a**), (Fig. **7**), 2,2,6,6-tetramethylpiperidine-4-hydroxy-*N*-oxyl (TEMPOL (**13b**), (Fig. **7**), and 2,2,6,6-tetramethylpiperidine-4-keto-*N*-oxyl (TEMPONE (**13c**), (Fig. **7**) at similar rates under both air and N_2 atmospheres. The ESR signal of the lipid-soluble spin probe 5-doxylstearate ((**13d**), (Fig. **7**) is stable on incorporation into erythrocytes from normal mice. In contrast, parasitized red cells reduce this nitroxide probe, at a rate which increases with the level of parasitemia. Inhibitors of electron transport such as KCN and NaN_3, increase the rate of reduction. It is proposed that nitroxide reduction occurs via the electron transport chain in the parasite. The antimalarial drug primaquine causes reduction of both water-soluble and lipid-soluble spin probes. This action of primaquine is independent of its ability to release H_2O_2 from oxyhemoglobin. The increased production of NADPH results in increased rates of reduction of the nitroxide radicals. Chloroquine, however has no such effect. Parasitized mice treated with chloroquine six hours prior to ESR measurements show less nitroxide reducing capacity than do untreated mice. The metabolic influences of the two antimalarial drugs are, thus, quite different.

Malaria parasites have been shown to be more susceptible to oxidative stress than their host erythrocytes. A chloroquine resistant malaria parasite, *Plasmodium falciparum* (FCR-3) was found to be susceptible *in vitro* to a pyridoxal based iron chelator-(1-[*N*-ethoxycarbonylmethyl-pyridoxylidenium]-2-[2'-pyridyl]hydrazine bromide (code named L2-9, (**14**) (Fig. **7**). 2 h exposure to 20 microM L2-9 was sufficient to irreversibly inhibit parasite growth. Desferrioxamine blocked the drug effect, indicating the requirement for iron. Oxygen however, was not essential. Spectrophotometric analysis showed that under anoxic conditions, L2-9-Fe(II) chelate undergoes an intramolecular redox reaction which presumably involves a one electron transfer and is expected to result in the formation of free radicals. Spin trapping coupled to ESR studies of L2-9-iron chelate showed that L2-9-Fe(II) produced free radicals both in the presence and absence of cells, while L2-9-Fe(III) produced free radicals only in the presence of actively metabolising cells [30].

Another group of molecules, quinones, are naturally occurring pigments in a variety of plants and fungi, and some are clinically important antitumor drugs [37]. They are substrates for flavoenzymes and can undergo either one- or two- electron reduction, a property of importance in determining the cytotoxic and antitumor effects of quinones [38-40].

The bioactivation of exogenous compounds with quinone structures, like many chemotherapeutic agents, has been demonstrated to proceed via one-electron reduction to semiquinone radicals, which, in a redox-cycle with the quinones under aerobic conditions, may reduce molecular oxygen to superoxide anions. Redox-cycling of quinones and their semiquinones is thought to be responsible for the concomitant oxygen toxicity often observed. Van de Straat *et al*. investigated the possible role of cytochrome P-450 in the one-electron reduction of quinoid compounds as well as

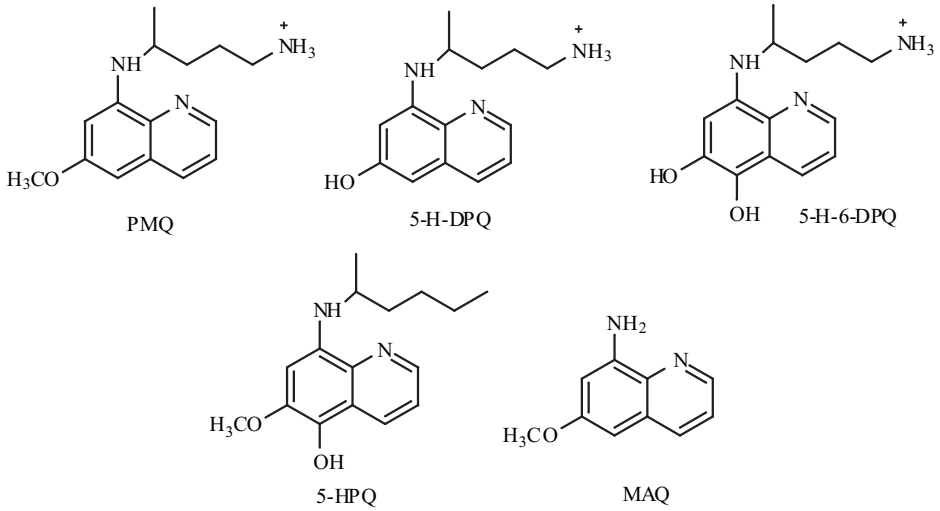

Fig. (8). Molecular structure of Primaquine metabolites with oxidative activity. PMQ = Primaquine; 5-HPQ = 5-hydroxyprimaquine; 5-H-DPQ = 5-hydroxydemethylprimaquine; 5-H-6-DPQ = 5,6-dihydroxydemethylprimaquine; MAQ = aminoquinoline.

in the formation of reduced oxygen species and used ESR combined with spin trapping to detect the free radicals formed [41]. Although menadione (2-methyl-1,4-naphthoquinone; vitamin K3 (**12a**) (Fig. **3**) is used therapeutically, it is also cytotoxic and causes a marked decrease of intracellular thiols such as GSH and protein sulfhydryl groups and the formation of $O_2^{\cdot -}$ in large amounts. Takahashi *et al*. [42] reported the identification of the transient semiquinone-type radicals by ESR spectroscopy during the non-enzymatic reaction of both menadione ((**12a**) (Fig. **3**) and 1,4-naphthoquinone with the biological reducing agents GSH and NADPH. The menadione-induced loss of cellular thiols occurs by their reaction with these active oxygen species and by the direct arylation of protein sulfhydryl groups and GSH. Reaction with GSH forms a menadione-GSH conjugate (2-methyl-3-S-glutathionyl-1,4-naphthoquinone; thiodione ((**12b**) (Fig. **3**) at the 3-position [43]. The authors observed ESR spectra obtained by reaction of both naphthoquinones and their GSH conjugates in boric acid-borax buffer, pH 8.5, under a nitrogen atmosphere, generated by the reaction of Menadione semiquinone with different concentrations of GSH and of thiodione with NADPH. The spectra proved to be identical. This experiment led the authors to discuss that the incubation of menadione or 1,4-naphthoquinone with the reducing agent, NADPH, led to the formation of the corresponding semiquinone-free radical in buffer at pH 7.4,8.5, or 9.0 under a nitrogen atmosphere. In the presence of GSH as a reducing agent, menadione and 1,4-naphthoquinone underwent conjugation with GSH at either or both of the 2- and 3-positions, depending upon the ratio of the quinone to GSH and net one-electron reduction to form the corresponding semiquinones. At lower GSH concentrations only reduction to the respective semiquinones was detected, as was reported for 1,4- naphthoquinone [44].

Artemisinin ((**11a**) (Fig. **3**) and its derivatives represent a very important new class of antimalarials; they are becoming more and more commonly used throughout the world. Artemisinin structure is unlike those of any other known antimalarial and is thus likely to have a different mechanism of action. The first clue to its mechanism came from synthetic chemists who demonstrated that the endoperoxide bridge was necessary for antimalarial activity [45,46]. Since peroxides are a known source of reactive oxygen species such as hydroxyl radicals and superoxide [47], this observation suggested that free radicals might be involved in the mechanism of action. The role of free radicals in the biological mechanism of action of artemisinin derivatives was demonstrated in the late 1980s [48].

The mechanism of action of these compounds appears to involve the heme-mediated decomposition of the endoperoxide bridge to produce carbon-centred free radicals. Reaction of the antimalarial and anti-schistosomal drug artemether (**11b**), an artesimnin derivative, (Fig. **3**) and catalytic amount of ferrous ion in the presence of excess cysteine gave two adducts of cysteine and previously postulated carbon-centred free radicals that were trapped with 2-methyl-2-nitrosopropane (MNP) and provided the very first direct evidence for the involvement of radicals in the *in vitro* cleavage of artemisinin-type compounds [49]. This piece of further evidence for the presence of carbon-centered radicals, especially the secondary carbon-centered free radical detected for the first time by the isolation of its coupling adduct, is helpful to understand the mechanism of action of artemether and other qinghaosu derivatives against parasites [50].

Two important pathogens of developing countries, *Mycobacterium leprae*, the etiologic agent of leprosy, and *Leishmania donovani*, the protozoal parasite that causes kalaazar, persist in the human host primarily in mononuclear phagocytes. The mechanisms by which they survive in these otherwise highly cytocidal cells are presently unknown. Since the best understood cytocidal mechanism of these cells is the oxygen-dependent system that provides lethal oxidants including the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^1O_2$), Chan *et al*. [51] sought specific microbial products of these organisms that might enable them to elude oxidative cytocidal mechanisms. Phenolic glycolipid I of *M. leprae* and lipophosphoglycan of *L. donovani* are unique cell-wall-associated glycolipids produced in large amounts by the organisms. In this study, phenolic glycolipid I derivatives and lipophosphoglycan were examined for their ability to scavenge potentially cytocidal oxygen metabolites *in vitro*. ESR and spin-trapping indicated that phenolic glycolipid I derivatives and lipophosphoglycan are highly effective in scavenging hydroxyl radicals and superoxide anions. The results suggested that complex glycolipids and carbohydrates of intracellular pathogens that can scavenge oxygen radicals may contribute to their pathogenicity and virulence [51].

The cytotoxins produced by phagocytic cells lacking peroxidases such as macrophages remain elusive. To elucidate macrophage microbicidal mechanisms *in vivo*, Linares *et al*. compared the lesion tissue responses of resistant (C57Bl/6) and susceptible (BALB/c) mice to *Leishmania amazonensis* infection. This comparison demonstrated that parasite control relied on lesion macrophage activation with inducible nitric oxide synthase expression (iNOS), nitric oxide synthesis, and extensive nitration of parasites inside macrophage phagolysosomes at an early infection stage. Nitration and iNOS expression were monitored by confocal microscopy; nitric oxide synthesis was monitored by ESR. The main macrophage nitrating agent was shown to be peroxynitrite-derived because parasite nitration occurred in the virtual absence of polymorphonuclear cells (monitored as peroxidase activity) and was accompanied by protein hydroxylation (monitored as 3-hydroxytyrosine levels). *In vitro* studies confirmed that peroxynitrite is cytotoxic to parasites whereas nitric oxide is cytostatic. The results indicated that peroxynitrite is likely to be produced close to the parasites and most of it reacts with carbon dioxide to produce carbonate radical anion and nitrogen dioxide whose concerted action leads to parasite nitration. In parallel, some peroxynitrite decomposition to the hydroxyl radical should occur due to the detection of hydroxylated proteins in the healing tissues. Consequently, peroxynitrite and derived radicals are likely to be important macrophage-derived cytotoxins [52].

While some groups studying parasitic diseases focus their work on free radical generation as the mechanisms of action, some have concentrated on the synthesis of new pharmaceutical agents by the coordination of antiprotozoal organic drugs [53]. Others have tested antitumor metal-

containing complexes such as cisplatin against kinetoplastid parasites [54]. These studies showed that the tested compounds display biological activity against protozoa, results that, along with the fact that many antiprotozoal drugs bind to DNA leads to expect that in general every DNA interacting compound could be active against parasites and that one of the mechanisms possible is through free radical generation [55].

Similar is the case of paramagnetic complexes $[Cu(dppz)(NO_3)]NO_3$ (**15**), $[Cu(dppz)_2(NO_3)]NO_3$ (**16**), $[Cu(dpq)(NO_3)]NO_3$ (**17**), and $[Cu(dpq)_2(NO_3)]NO_3$ (**18**) (dppz: dipyrido[3,2-a:2',3'-c]phenazine; dpq: dipyrido[3,2-a:2',3'-h]quinoxaline) whose molecular structure was characterized by ESR, among other spectroscopies [56]. DNA interaction studies showed that intercalation is an important way of interacting with DNA for these complexes. The biological activity of these copper complexes was evaluated on *Leishmania braziliensis* promastigotes, and the results showed leishmanicidal activity. Preliminary ultrastructural studies with the most active complex (**16**) at 1 h revealed parasite swelling and binucleated cells. This finding suggests that the leishmanicidal activity of the copper complexes could be associated with their interaction with the parasitic DNA, results in agreement with the ESR results indicating that the spectra for complexes (**15**) and (**17**) showed a high symmetry in the plane of the copper, which is indicative of a high electron density in the copper plane. These results lead to propose a square planar structure for those complexes. The spectrum of complex (**16**) showed an asymmetry in the plane of the copper and the hyperfine splitting resolution is almost totally lost. The spectrum of compound (**18**) showed a slight distortion of the planar symmetry, with loss of resolution in the lines corresponding to the hyperfine splitting.

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