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Antimicrobial Action of Chelating Agents: Repercussions on the Microorganism Development, Virulence and Pathogenesis

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Abstract: Infections caused by resistant microorganisms often fail to respond to conventional therapy, resulting in prolonged illness, increased treatment costs and greater risk of death. Consequently, the development of novel antimicrobial drugs is becoming more demanding every day since the existing drugs either have too many side-effects or they tend to lose effectiveness due to the selection of resistant strains. In view of these facts, a number of new strategies to obstruct vital biological processes of a microbial cell have emerged; one of these is focused on the use of metal-chelating agents, which are able to selectively disturb the essential metal metabolism of the microorganism by interfering with metal acquisition and bioavailability for crucial reactions. The chelation activity is able to inhibit the biological role of metal-dependent proteins (e.g., metalloproteases and transcription factors), disturbing the microbial cell homeostasis and culminating in the blockage of microbial nutrition, growth and development, cellular differentiation, adhesion to biotic (e.g., extracellular matrix components, cell and/or tissue) and abiotic (e.g., plastic, silicone and acrylic) structures as well as controlling the *in vivo* infection progression. Interestingly, chelating agents also potentiate the activity of classical antimicrobial compounds. The differences between the microorganism and host in terms of the behavior displayed in the presence of chelating agents could provide exploitable targets for the development of an effective chemotherapy for these diseases. Consequently, metal chelators represent a novel group of antimicrobial agents with potential therapeutic applications. This review will focus on the anti-fungal and anti-protozoan action of the most common chelating agents, deciphering and discussing their mode of action.

Keywords: Alternative chemotherapy, antimicrobial activity, chelating agents, growth, differentiation, fungi, Interaction, opportunistic infections, pathogenesis, protozoa, virulence.

1. INTRODUCTION

Microorganisms are the cause of many infectious diseases in both immunosuppressed and immunocompetent individuals. In the category of pathogenic microorganisms are included bacteria, fungi and protozoa. Natural or human-triggered changes in the environment might upset the natural balance between living organisms. These new environmental conditions may benefit microbial pathogens, allowing them to multiply rapidly and increase the risk of exposing humans who share that environment. Infections with resistant microorganisms cause high rates of morbidity and mortality and also increase health care treatment costs [1-3]. Antimicrobial resistance is a persistent worldwide healthcare concern. In this context, the development of novel antimicrobial agents is needed to treat and/or improve the clinical outcome of microbial infected patients. This theme is more problematical regarding the infections caused by both protozoa and fungi, whose clinical treatment is extremely difficult due to the eukaryotic architecture of these microorganisms, which are similar to host cells.

Several diseases and physiological disorders are frequently related with the excess or accumulation of certain essential metals in the organism, including some microbial infections [4, 5]. Specific metals are active constituents of many essential biomolecules (e.g., metalloproteins) for the normal functioning of the organism and the correct activity of all its metabolic and physiological processes, controlling the cellular homeostasis. In this sense, transition metal ions like iron, copper, zinc, manganese, cobalt and nickel are essential nutrients to all forms of life, presenting unique chemical and physical properties that make them attractive molecules for use in biological systems. Besides, several other metals like sodium, potassium, magnesium and calcium are essential components of a variety of biological systems, participating in a plethora of crucial functions in a cell [6]. Although metals all have a specific biological functions essential for life, lack of regulation of intracellular metal pools can lead to toxicity often through the formation of oxygen radicals (including the hydroxyl radical) via the Fenton series of reactions [6]. Therefore, chelation therapies may be especially useful and adequate for the treatment of imbalances such as these. Specifically iron deficiency is the most frequent nutritional problem; however, iron overload is the most common metal toxicity condition and has the highest mortality rate worldwide [7-9]. Iron overload is known to exacerbate many infectious diseases, such as microbe infections [10, 11]. In microorganisms, the importance of well-defined amounts of iron for the survival and cell growth is well established [12]. To cause infection, protozoa, fungi and bacteria must sequester growth-essential iron from their hosts [13-15]. Moreover, iron is important for the virulence of the majority of microorganisms, and the function of the genes regulating iron uptake is coupled with the manifestations of the virulence phenotype [14, 16, 17]. Most microorganisms produce small molecules, the siderophores, which have a high affinity for iron(III). These specific chelators are synthesized by microbes for the uptake and transfer of essential iron from the environment or the host cells. The main classes of siderophores are the hydroxamate siderophores, including deferrioxamine B (DFO) and the catechol siderophores [18]. The iron chelating proteins (transferrin and lactoferrin) have been evolved for the iron acquisition in mammalian cells via specific pathways, and these may prevent the growth of microbial pathogens and their proliferation in host cells [19, 20]. Iron chelating agents and other drugs with chelating properties (tetracyclines, anthracyclines, salicylates and hydroxyurea) can affect the iron uptake by microbial pathogens and, accordingly, can either inhibit or promote microbial growth

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and proliferation [21]. Considering infections, findings available in the literature show that either iron deficiency may favor the proliferation of parasites, once such deficiency leads to damage of the immune system, or alternatively, iron deficiency may favor the control of infection, as it limits the amount of iron available to the parasite, illustrating a delicate balance between iron availability and microorganism infection [22-26]. Moreover, iron chelation alone, or combined with classical antimicrobial drugs, may be useful for prevention and treatment of microbial infection.

Chelation therapy is approved for treating lead poisoning and heavy metal toxicity (e.g., aluminum, mercury and cadmium) [27, 28] by the United States of America Food and Drug Administration (FDA).For instance, exposure to Al^{3+} ions can cause renal failure and aluminum metal dust gives rise to respiratory and central nervous system symptoms. The recommended chelation treatment is DFO and 1,2-dimethyl-3-hydroxypyrid-4-one [27]. Acute inhalation of cadmium metal dust promotes respiratory and gastrointestinal disorders. Lung, liver and kidney damage arises from exposure to relatively small quantities of Cd^{2+} ions. Although there is no proven benefit, some clinicians recommend chelation therapy with $CaNa_2EDTA$ [28]. For patients chronically exposed to cadmium administration of dimercaprol and substituted dithiocarbamates seems beneficial [28]. Chronic inhalation of mercury vapor and ingestion of mercury compounds causes severe neurological effects, interstitial pneumonitis and death. Amongst the sulfur-containing ligands that are recommended for treatment

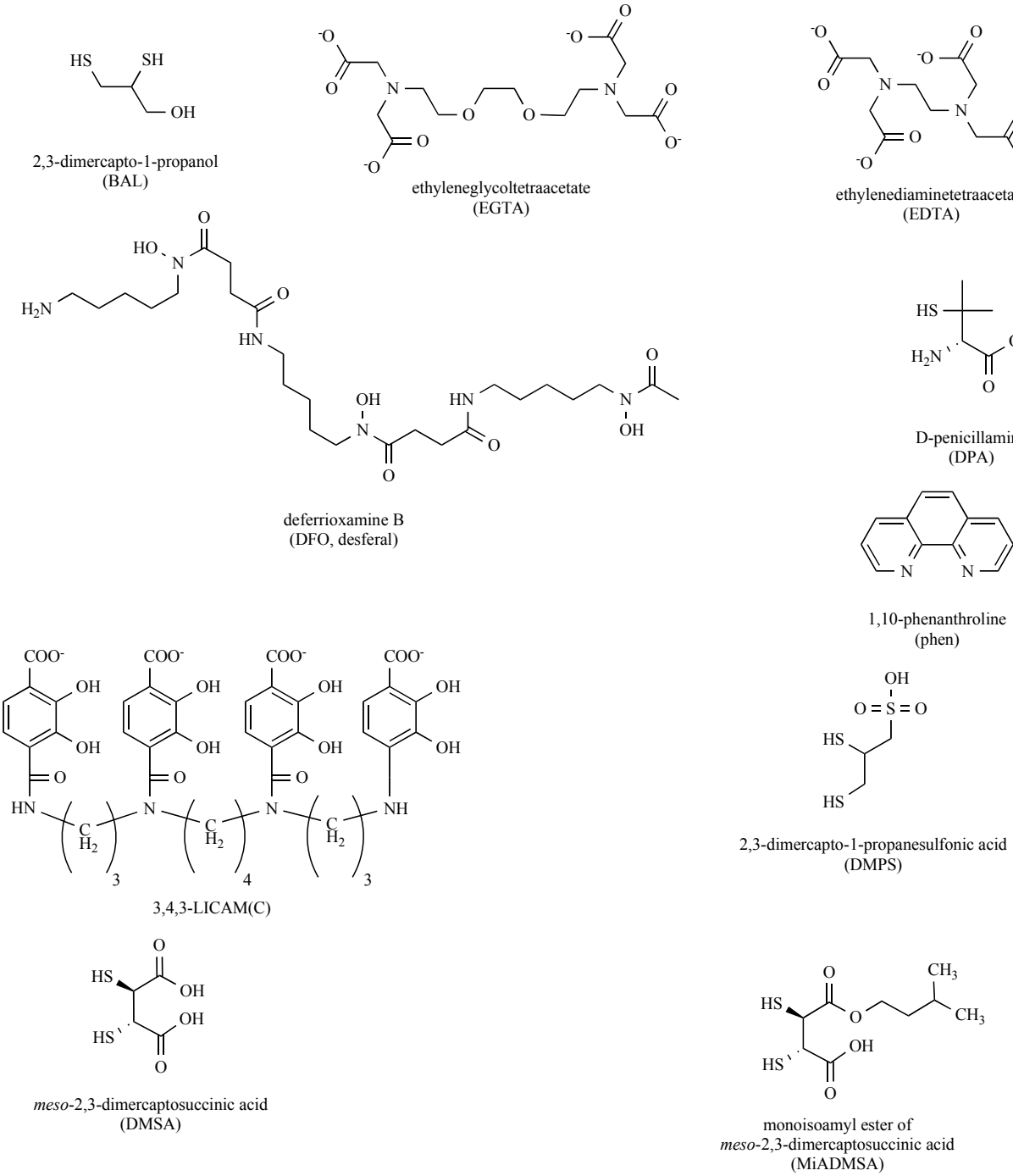

Fig. (1). Molecular structure of the chelating ligands: BAL; EGTA; EDTA; DFO; DPA;PHEN; 3,4,3-LICAMC; MiADMSA; DMSA and DMPS.

are 2,3-dimercaptopropane-1-sulphonic acid (DMPS) and 2,3-dimercapto-1-propanol (British-Anti-Lewisite, BAL). Additionally, this therapy is known to be effective in reducing the toxic build-up of metal ions arising from physiological disorders such as Wilson's disease (copper) [29-31] and thalassemia (iron) [32]. Common chelating agents for detoxification after metal poisoning are shown in Fig. (**1**). including (i) ethylenediaminetetraacetic acid (EDTA) that is known to effectively detoxify Ca^{2+} and Pb^{2+} [33], (ii) BAL following Hg^{2+}, As^{3+}, Sb^{3+} and Ni^{2+} exposure [34], (iii) DFO for detoxification of Fe^{3+} and Al^{3+} [35], (iv) D-penicillamine (DPA) [36] for coordination to both Cu^{2+} and Hg^{2+}, and (v) 3,4,3-LICAMC [37] for treatment following exposure to the radionuclide Pu^{4+} [38, 39].

The main goal of chelation treatment is to transform the offending toxic metal ion that is ligated by biological ligands, into a new, non-toxic complex, which can be safely excreted from the organism [28, 40]. For example, the treatment of Wilson's disease, resulting from an excess build-up of copper in the body, involves the use of the chelating agent DPA (Fig. **1**) which forms a soluble complex with copper ions that is colored an intense purple and, surprisingly, has a molecular mass of 2600 amu [36]. Another surprising finding is that the complex will not form unless chloride or bromide ions are present and the isolated complex always contains a small amount of halide. These puzzling facts were explained when the X-ray crystal structure was characterized (Fig. **2**), where the structure consists of a central halide ion surrounded by eight Cu^{+} atoms bridged by sulfur ligands. These are in turn coordinated to six Cu^{2+} atoms surrounded by the chelating amino groups of the DPA ligand. The profile of a successful chelating drug includes high affinity for the toxic metal(s) but low affinity for essential metals, minimal toxicity, lipid solubility and good absorbability. Age-related differences in efficacy of chelation therapy should also be considered [37, 41]. Further applications for chelation therapy have also been postulated as potential alternatives in the prevention/treatment against cardiovascular disease [42, 43], autism [44, 45], diabetes [46], osteoporosis [47, 48], atherosclerosis [49, 50], Parkinson's disease [51, 52] and certain types of cancer [53, 54]. Metal ion chelation has also been suggested as a prospective therapy for several microbial infections [55-57].

Herein, we review the ability of chelating agents to block essential eukaryotic microbial (fungi and protozoa) physiological processes like nutrition, growth and development, cellular differentiation, adhesion to both biotic and abiotic structures, as well as their capacity to control the *in vivo* infection progress in animal models. The synergistic action of chelating agents and classical antimicrobial compounds will also be presented and discussed.

2. ANTI-TRYPANOSOMATIDAL ACTION OF CHELATING AGENTS

Diseases caused by human trypanosomatids, such as leishmaniases (etiologic agents: *Leishmania* spp.), sleeping sickness (*Trypanosoma brucei* complex) and Chagas' disease (*Trypanosoma cruzi*), continue to pose a serious threat to human health around the world [58]. Furthermore, the current therapeutic arsenal against the human pathogenic trypanosomatids is clearly inadequate, extremely limited and all current treatments suffer from significant drawbacks including parenteral route of administration, length of treatment, toxicity and/or cost, which limits their utilization in disease endemic areas and emphasizes the urgent need to develop new effective, safe and cost-effective drugs [59].

2.1. Iron Chelators

Iron is vital for all trypanosomatid parasites and plays a significant role in pathogenesis and immune control of these organisms. In this sense, depletion of this essential nutrient in trypanosomatids rapidly decreases the rate of DNA synthesis, increases the oxidative stress levels via loss of superoxide dismutase and ascorbate-dependent peroxidase activity, blocks the J-base synthesis and stops electron transfer to the alternative oxidase, leading inexorably to the death of the protozoan [23]. Various studies have established that administration of DFO in *T. cruzi* infection modifies the course of Chagas' disease, reducing parasitemia and mortality [60-65].

The first reports of a correlation between iron levels and the development of infection by *T. cruzi* were published by Lalonde and Holbein [62] and Loo and Lalonde [61]. Those authors observed that the depletion of iron stores in the liver of C57BL/6 mice that had been treated with DFO on the $5^{th}/6^{th}$ day post-infection or maintained on an iron-deficient diet reduced the parasitemia and mortality of the infection. Furthermore, Lalonde and Holbein [62] demonstrated that no significant changes occurred in the serum iron levels of non-infected mice that had been either

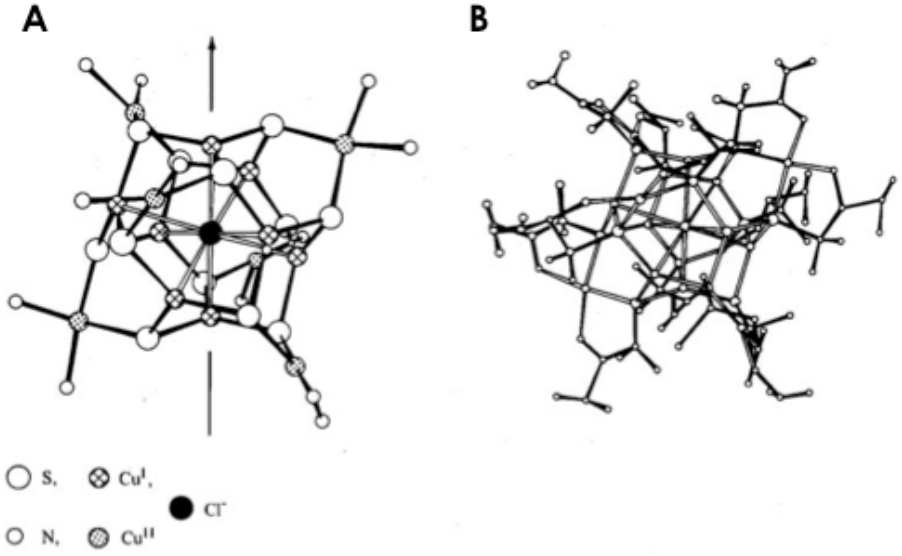

Fig. (2). Molecular structure of the copper complex of D-penicillamine. The $[Cu^{+}_{8}Cu^{2+}_{6}$ (penicillaminate)$_{12}Cl]$ ion: (**A**) the central cluster of Cu and ligating atoms only; (**B**) the entire ion with the central cluster oriented as in (**A**).

treated with DFO, while treated/*T. cruzi*-infected animals presented iron supplies that were sufficient to maintain a normal immune response.

Subsequently, Pedrosa and co-workers [63] evaluated the effects of iron deficiency on the evolution of experimental *T. cruzi* infection in CFW mice and observed a strain-dependency. Compared to the control group, mice infected with the YuYu strain developed a less severe form of the disease when treated with DFO at a dose of 10 mg/mouse on the 5thday post-infection, but no differences were observed in mice infected with Y and CL strains. In this sense, these first studies concerning the effect of DFO on the development of *T. cruzi* infection have typically involved short-term treatments. Nonetheless, considering that DFO is rapidly cleared from the circulation [66], it is suggested that a longer period of treatment with this chelator could give rise to a more pronounced effect on the development of *T. cruzi* infection. In order to test this hypothesis, Arantes and co-workers [64] showed that Swiss male mice experimentally infected with *T. cruzi* Y strain and submitted to a DFO prolonged treatment (from 14 days before infection until the 14th or 21stday post-infection) gained in a powerful protection against infection, leading to attenuation of parasitemia and reduction of the mortality rate in the infected mice. Interestingly, infected/treated mice presented lower levels of iron in the liver compared with treated/non-infected and control animals, while the serum iron levels of the infected/non-treated group were higher on the 21st day post-infection in comparison with control and infected/treated groups, which may suggest that decrease of iron in the host leads to *T. cruzi* infection attenuation. In addition, the reduced concentration of iron found in the liver of infected mice was probably associated with the regulation of iron within the organism as a response to infection by *T. cruzi*. The lower serum iron levels in the infected/treated group compared with the infected/non-treated group after 21 days would then correlate with the chelation of plasma iron by DFO.

While the results obtained, using a murine model, have shown that there were reductions in both the number of circulating parasites and in mortality, it was also observed that the animals did not suffer from anemia [64, 65]. It was concluded, therefore, that DFO had a direct effect on the parasite and not on the reduction of iron supply in the host. In this sense, Francisco and co-workers [67] showed that DFO possesses the capacity to enhance antioxidant activity in *T. cruzi*-infected Swiss male mice, particularly by up-regulating superoxide dismutase levels, but it also increases oxidative stress, which exerts pressure on the parasite, over a long period of time. This indicates that the mode of action of the drug involves a positive contribution to the host together with an effect that is not beneficial to the parasite, which may represent a possible mechanism in the reduction of parasitemia.

In a distinct approach, the effects of benznidazole (BZ) therapy, the only drug currently available in Brazil for the treatment of Chagas' disease, in combination with DFO on the development of infection in Swiss male mice inoculated with *T. cruzi* Y strain have been investigated [65]. Therapy with BZ for 21 days promoted a decrease in parasitemia levels and in mortality rates irrespective of the presence of DFO, but BZ in combination with DFO treatment for 35 days (BZ/DFO-35) gave a 100% survival level, suggesting that iron levels could influence the course of infection. All infected groups presented lower levels of iron in the liver, but serum iron concentrations were greater in DFO-35 and BZ/DFO-35, whereas hemoglobin levels were higher in BZ/DFO-35 and lower in DFO-35 compared with other treated groups. The percentage cure, determined from negative hemoculture and polymerase chain reaction results in animals that had survived for 60 days post-infection, was 18% for BZ and BZ/DFO-35, 42% for BZ combined with DFO for 21 days, and 67% for DFO-35, which reveals that, when employed in combination with BZ, the chelator DFO is more effective if administered for a shorter period of time. The fact that the DFO-35 group showed a 67% cure rate is strong evidence of the protective effect of DFO in the surviving animals. These results demonstrated in a quantitative manner the effectiveness of combination therapy with both drugs in comparison with DFO treatment alone. In summary, the findings of Pedrosa and co-workers [63], Arantes and co-workers[64] and Francisco and co-workers[67] suggest that the effect of DFO is dependent on whether the therapy is initiated pre- or post-infection and on the length of time over which administration is continued.

One different group of iron chelator that has activity *in vitro* against *T. cruzi*are the aminothiol ligands, which have a predicted ability to bind iron, are lipophilic and have anti-oxidant properties. The anti-trypanosomal activity of three aminothiol derivatives have been examined: diethyl and dimethyl forms of ethane-1,2-bis (*N*-1-amino-3-ethylbutyl-3-thiol) (BAT-TM and BAT-TE), which are tetradentate ligands; and *N′*,*N′*,*N′*-tris(2-methyl-2-mercaptopropyl) 1,4,7-triazacyclononane (TAT), a hexadentate ligand [68]. TAT had an IC_{50}value of 52 μM against epimastigote forms, while BAT-TM and BAT-TE were inhibitory only at concentrations of>250 μM. The inhibitory activity of TAT against epimastigotes in this assay was less than that of the control drug, pentamidine, but greater than that reported for other iron chelators, including *N*-alkyl derivatives of 2-ethyl-and 2-methyl-3-hydroxypyrid-4-ones, *N*,*N′*-bis(*o*-hydroxybenzyl)-(±)-*trans*-1,2-diaminocyclohexane, cyclotetrachromotropylene and carboxy derivatives of pyridine, pyrazine and pyrazole [69].

In *T. cruzi*-infected Swiss mice, BAT-TE and BAT-TM had no anti-trypanosomal activity in doses up to 200 mg/kg, whether the route of administration was intraperitoneal or oral. Interestingly, BAT-TE at a concentration of 270 μM completely arrested the growth of trypomastigote forms in mouse blood stored at 4^{o}C for 24 h (IC_{50}= 67.7 μM), while BAT-TM arrested growth at 630 μM (IC_{50}= 158 μM) and TAT at concentrations >800 μM (IC_{50} =415 μM). In addition to the 24h experiment, parasites were cultivated in the presence of 60 μM concentrations (25 mg/ml) of either BAT-TE or BAT-TM for 3 weeks. The samples of infected stored mouse blood were totally free of trypomastigotes by the end of the experiment. The discrepancy observed in the activity pattern of BATs and TAT against different stages of the parasite may reflect the fact that TAT, in contrast to the less lipophilic BAT derivatives, tends to precipitate at 4^{o}C, the temperature at which the experiments with trypomastigotes in stored blood were performed. The trypanocidal activity of BAT derivatives in blood stored at 4^{o}C makes these compounds potential candidates for the purpose of clearing donated blood of trypomastigotes [68].

The degree of inhibition found for TAT against *T. cruzi* epimastigote forms is comparable with the results obtained previously by Rodrigues and co-workers [70] for some metal chelators. In the latter study, the selection of the chelating agent for testing against *T. cruzi* epimastigotes was based on the structural factors that govern the ease with which chelating agents can gain access to intracellular sites. The screening experiments revealed that all the dithiocarbamates (or carbodithioates) that contain only nonpolar substituents attached to the nitrogen of the dithiocarbamate function were active, and in some cases the ability to inhibit growth greatly exceeded by far the action of BZ (46% inhibition at 5 μg/ml). Similar results were found for thiuram disulfides, which were selected for inclusion because of their partial *in vivo* transformation to dithiocarbamates. Treatment of epimastigotes with sodium diethyldithiocarbamate has been shown to increase the concentration of both the superoxide ion and hydrogen peroxide by more than 10-fold [71], which suggests a possible common mode of action of both the dithiocarbamates and the thiuram disulfides via inactivation of the superoxide dismutase after removal of the iron ions that are essential to its activity. The resultant increase in both of the reactive oxygen species is thought to be responsible for the inhibition observed. In addition, the

pronounced trypanocidal activity (higher than 70%) of *N,N,N′N′*-tetrakis(2-pyridylmethyl) ethylenediamine is attributed to the combination of a considerable degree of lipophilicity with its high-stability constants with essential metal ions, which enable it to both penetrate cellular membranes and tie up iron, zinc and copper within thermodynamically stable complexes.

An interesting approach concerning the effects of iron chelators in *Leishmania* spp. was explored by Zarley and co-workers [72]. After macrophage phagocytosis, it is well documented that, in order to successfully establish an infection, promastigotes must survive despite the local production of toxic oxidant species, such as superoxide and hydrogen peroxide [73]. Hydrogen peroxide can be converted to the hydroxyl radical (˙OH) through the Fenton reaction in the presence of a source of iron: $H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$. The ˙OH is a highly toxic species and has been implicated in the killing of microorganisms by phagocytes, as well as phagocyte-associated inflammation and tissue injury [73]. As a means of investigating a possible role of ˙OH in H_2O_2-mediated killing of *Leishmania chagasi*, promastigotes were pre-incubated in different concentrations of DFO before exposure to H_2O_2. Increasing concentrations of DFO resulted in increasing protection from H_2O_2-mediated toxicity, which suggests that the increased resistance was indeed due to the iron-chelating capacity of DFO.

One of the first studies employing DFO against *Leishmania* spp. was performed *in vitro* against *Leishmania donovani* promastigotes. It was found that DFO was inactive at 50 μg/ml for 3 days. On the other hand, 44% and 60% of amastigotes were eliminated when macrophages infected with *L. donovani* were exposed to 50 μg/ml of free or liposome-encapsulated DFO, respectively [74]. Treatment of BALB/c mice with intraperitoneal injections of DFO resulted in a slight delay of the development of cutaneous lesions by *Leishmania major*. In contrast, systemic iron delivery in the form of intraperitoneal injections of iron-dextran, at early time points of parasite delivery, significantly limited footpad pathology [75]. The effect of iron supplementation appears to be due to its effect on the immune response of the host, rather than to any direct effect on the parasite [75, 76]. In the later stages, there is activation of NF-κB and an increased number of interferon (IFN)-γ positive $CD4^+$ T-cells are recruited to the draining lymph node. Thus, in this system iron probably mediates its effect via reactive oxygen species signaling through NF-κB, leading to a sustained TH_1response against the parasites [75, 76].

The effect of DFO and hydroxypyridin-4-ones in *Leishmania infantum*and *L. major*growth *in vitro* was compared by Soteriadou and co-workers [77]. The results demonstrated that iron chelators of different chemical classes inhibit the growth of *Leishmania* spp. promastigotes, and of particular interest was the finding that hydroxpyridin-4-ones are more potent inhibitors of *Leishmania* growth than DFO: the IC_{50} values were 150 μM, 75 μM and 50 μM for DFO, 1,2-dimethyl-hydroxypyridin-4-one (L1) and 1,2-diethyl-hydroxypyridin-4-one (CP94), respectively. Taking into consideration that the ligand:metal ratio in the L1/Fe and CP94/Fe complexes is 3:1 and that of DFO/Fe is 1:1, it appears that L1 and CP94 are approximately 12 and 18 times, respectively, more inhibitory than DFO. This might imply that the degree of lipophilicity of the compound is an important factor in determining the extent to which the iron chelator crosses cell membranes and mobilizes iron from cells.

Malafaia and co-workers [52] evaluated the effect of iron deficiency, induced by DFO, on the course of the infection by *L. chagasi* in BALB/c mice. A significant decrease in hemoglobin blood levels were found in infected/treated animals in comparison to infected/non-treated groups after 4 and 6 weeks of infection. In addition, DFO was able to provoke a significant reduction of liver and spleen parasitic load when the evaluations were performed 4 and 6 weeks after infection, respectively, which coincides with the peak of parasitism in each organ. Significant differences were not observed in the production of IFN-γ and interleukin (IL)-4 among the experimental groups. The lowering of the parasite numbers suggested that iron deficiency may contribute to control the murine infection with *L. chagasi*.

Iron has also been implicated in the activity of leishmanicidal drugs. Using DFO, Mehta and Shaha [78] showed that iron depletion inhibits changes in the mitochondrial membrane potential and ATPase activity produced by antimonial or arsenical drugs. This inhibition can be reversed if the DFO is saturated with iron prior to incubation with the parasites, showing that the effect is dependent on iron chelation. In addition, it has been demonstrated that DFO can reduce the level of cell death induced by treatment with SbIII or AsIII, whereas addition of iron causes a slight exacerbation of cell death. The drug-induced death of *Leishmania* spp. may involve activation of their programmed cell death (apoptosis-like process) at the mitochondrial level, and exacerbation of this process by iron is likely to reflect increased oxidative stress resulting from ˙OH generated by the superoxide-driven Fenton reaction.

Unlike *T. cruzi* and *Leishmania* spp., *Trypanosoma brucei*(the causative agent of African trypanosomiasis or sleeping sickness in humans) lives and multiplies extracellularly in the blood and tissue fluids of the mammalian host. Like all living organisms, *T. brucei* requires iron for growth [79].In the case of bloodstream forms of *T. brucei*, iron is delivered by host transferrin, the uptake of which is mediated by a transferrin receptor [80, 81]. The effect of DFO on culture-adapted bloodstream forms of *T.brucei* TC 221 was studied in comparison with that on mouse myeloma HyAg8 cells and human HL-60 cells [82]. Compared with these mammalian cells, bloodstream forms of *T. brucei* are 10-fold more sensitive to iron depletion. The primary target of the chelator is obviously the intracellular iron, as the toxicity of DFO was abolished by addition of iron-saturated chelator and/or the presence of holotransferrin. To identify probable target sites, the effect of DFO on ribonucleotide reductase, alternative oxidase and superoxide dismutase, three iron-dependent enzymes in bloodstream-form trypanosomes, was also studied. Incubation of the parasites with the chelator leads to inhibition of DNA synthesis and lowers oxygen consumption, indicating that DFO may affect ribonucleotide reductase and alternative oxidase. The compound does not inhibit the holoenzymes directly but probably acts by chelating cellular iron, thus preventing its incorporation into the newly synthesized apoproteins. Treatment of the parasites with DFO for 24 h has no effect on the activity of superoxide dismutase, which may be due to the long half-life of the enzyme. DFO applied intraperitoneally to *T. brucei*-infected mice at a daily dosage of 300 mg/kg up to the 26^{th}day post-infection had only a slight effect on the course of parasitemia compared with infected control mice. The failure of DFO to clear the parasites from the blood of the host is probably due to its short half-life *in vivo* [82].

In another study, the effect of 13 metal chelators on the growth of *T. brucei*, *Trypanosoma congolense* (the causative agent of nagana cattle disease) and human HL-60 cells was tested *in vitro* [83]. With the exception of 5-sulfosalicylic acid and dimethylglyoxime, all other chelators exhibited anti-trypanosomal activities, with IC_{50} values ranging between 2.1-220 μM. The most trypanocidal chelators were DFO, 1,10-phenanthroline and its 4,7-diphenyl derivative (an iron chelator) and 2,9-dimethy-4,7-diphenyl derivative (bathocuproine, a copper chelator) and the iron chelator, 8-hydroxyquinoline, with IC_{50} values in the micromolar range. Generally, *T. congolense* was somewhat less susceptible to the compounds tested than *T. brucei*. Except for bathocuproine and quercetin, all other chelators that were active against trypanosomes also displayed cytotoxicity towards human HL-60 cells, with IC_{50} values ranging from 6.2 μM to 97 μM. Therefore, the IC_{50} ratios of cytotoxic/trypanocidal activity (selectivity index) were found to be

in a modest range for all compounds. Only DFO and 4,7-diphenyl-1,10-phenanthroline gave IC_{50} ratios between 10 and 30. For comparison, commercially available drugs used for treatment of sleeping sickness and nagana have significant higher selectivity indices (1944 for suramin and 692 for diminazene aceturate). It seems that the lipophilicity of iron-chelating agents is the crucial factor and may be taken into consideration in the selection of novel anti-trypanosomal drugs.

When the structural and cell biological characterization of the three monothiol glutaredoxins (1-C-Grx) in the different life and growth stages of African trypanosomes was performed, the essential role of the mitochondrial 1-C-Grx1 in the iron metabolism of these parasites was determined [84]. The protein 1-C-Grx1 can coordinate one [2Fe-2S] center/protein dimer using glutathione as the non-protein ligand. Overexpression of an ectopic copy of *1-c-grx1* resulting in a 5–10-fold concentration, compared with the authentic protein, did not affect the proliferation rate and morphology of bloodstream cells when grown under optimal conditions. Interestingly, overexpression of 1-C-Grx1 in *T. brucei* resulted in an increased sensitivity of the parasite toward the DFO. The unphysiologically high concentration of 1-C-Grx1 may augment the depletion of the "free iron" pool due to an increased synthesis of iron-sulfur cluster proteins and/or to iron sequestration in the form of the [Fe-S]-1-C-Grx1 complex. The 2-fold down-regulation of 1-C-Grx1 in wild-type bloodstream parasites upon treatment with DFO could thus be a compensatory mechanism to balance the cellular iron levels. Overexpression of 1-C-Grx1 also sensitized trypanosomes against H_2O_2-mediated oxidative stress: exposure of these cells to H_2O_2, but not to iron, impaired cell growth, while treatment of wild-type bloodstream parasites with H_2O_2 caused a 2-fold up-regulation of 1-C-Grx1. Although the underlying molecular mechanisms are not yet known, it is clear that overexpression of 1-C-Grx1 lowers the capacity of bloodstream cells to withstand an exogenous H_2O_2 challenge. In this sense, the authors provided strong evidence that mitochondrial 1-C-Grx1 is an abundant protein that plays a crucial role in the iron and redox homeostasis of the parasite [84].

2.2. Zinc Chelators

The *in vitro* effects of 1,10-phenanthroline on the ultrastructure and growth of *T. cruzi* Y strain were investigated [85]. 1,10-Phenanthroline effectively inhibited the proliferation of *T. cruzi*epimastigote forms at concentrations as low as 2.5 µg/ml. Epimastigotes displayed swelling and electron-dense deposits in the kinetoplast, mitochondrion and cisternae of the endoplasmic reticulum. These morphological alterations were dose-dependent and first appeared at a concentration of 5 µg/ml. Analytical electron microscope examination indicated that the metallic portion of the electron-dense deposits was predominantly calcium. Natural populations of *T. cruzi* are very heterogeneous in biological, biochemical, immunological and molecular features [86]; consequently, different strains from endemic areas might be responsible for distinct clinical manifestations and chemotherapy response [87]. Take this into consideration, we tested the effect of 1,10-phenanthroline in three strains belonging to distinct phylogenetic lineages of *T. cruzi*: Dm28c (TCI), Y (TCII) and 4163 (Z3). All these strains had their cellular growth powerfully inhibited by 1,10-phenanthroline after 96 h of incubation (Fig. **3A**).

Inhibition of metalloproteases by 1,10-phenanthroline occurs by removal and chelation of the metal ion required for catalytic activity, leaving an inactive apoenzyme. 1,10-Phenanthroline targets mainly zinc-metalloproteases, with a much lower affinity for calcium [88, 89]. Lowndes and co-workers [90] reported for the first time the expression of a complex array of metalloproteases in *T. cruzi*, showing considerable qualitative and quantitative variation in different strains and developmental stages of the parasite.

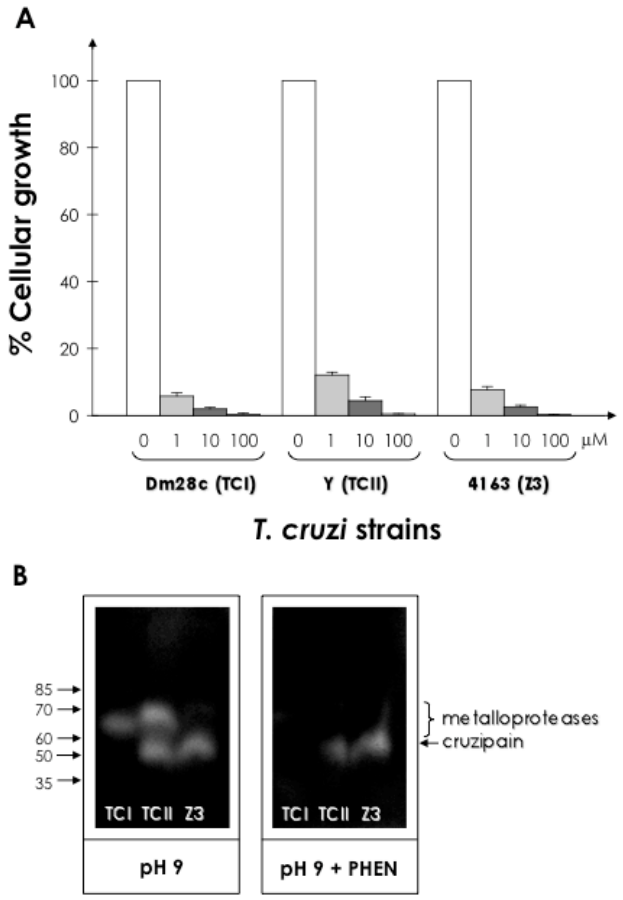

Fig. (3). Effect of PHEN on the growth of different phylogenetic strains of *Trypanosoma cruzi*: TCI (Dm28c), TCII (Y) and Z3 (4163) lineages. (**A**) Epimastigotes were grown for 96 h in the presence of different concentrations (0, 1, 10 and 100 µM) of PHEN. For experimental details see Sangenito and co-workers [282]. (**B**) Gelatin-SDS-PAGE showing the alkaline cellular protease profiles of *T. cruzi* strains after incubation the gels in the absence or presence of PHEN. Numbers on the left indicate the relative molecular mass of the markers. Note the presence of a 40 kDa protease that corresponds to the major cysteine protease (cruzipain) produced by this trypanosomatid and metalloproteasesin the range of 60-70 kDa. For experimental details see Fampa and co-workers [283] and Santos and co-workers [284].

Metalloproteases inhibited by 1,10-phenanthroline were already reported in *T. cruzi*, including: a family of *gp63* genes [91, 92] homologous to the gp63 of *Leishmania* spp. that are important for host cell infection [93],two metallocarboxypeptidases belonging to the M32 family previously found only in prokaryotes [94] and matrix metalloprotease-9-like proteins [95]. It is possible to detect metalloprotease activity in cellular extracts derived from *T. cruzi* epimastigotes by gelatin-containing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. **3**). Due to its high biochemical diversity, *T. cruzi* strains produced different amounts of metalloproteases sensible to 1,10-phenanthroline (Fig. **3B**).

Bonaldo and co-workers [96] investigated the role of 1,10-phenantholine, among other protease inhibitors, upon *T. cruzi* metacyclogenesis. In order to do so, clone Dm28c epimastigotes were incubated in chemically defined conditions in which epimastigotes transform into metacyclic trypomastigotes. In the presence of the metal chelator, cells displayed a normal mobility, but differentiation was blocked in a dose-dependent manner, reaching 85% inhibition when the chelator was added at 10 µM.

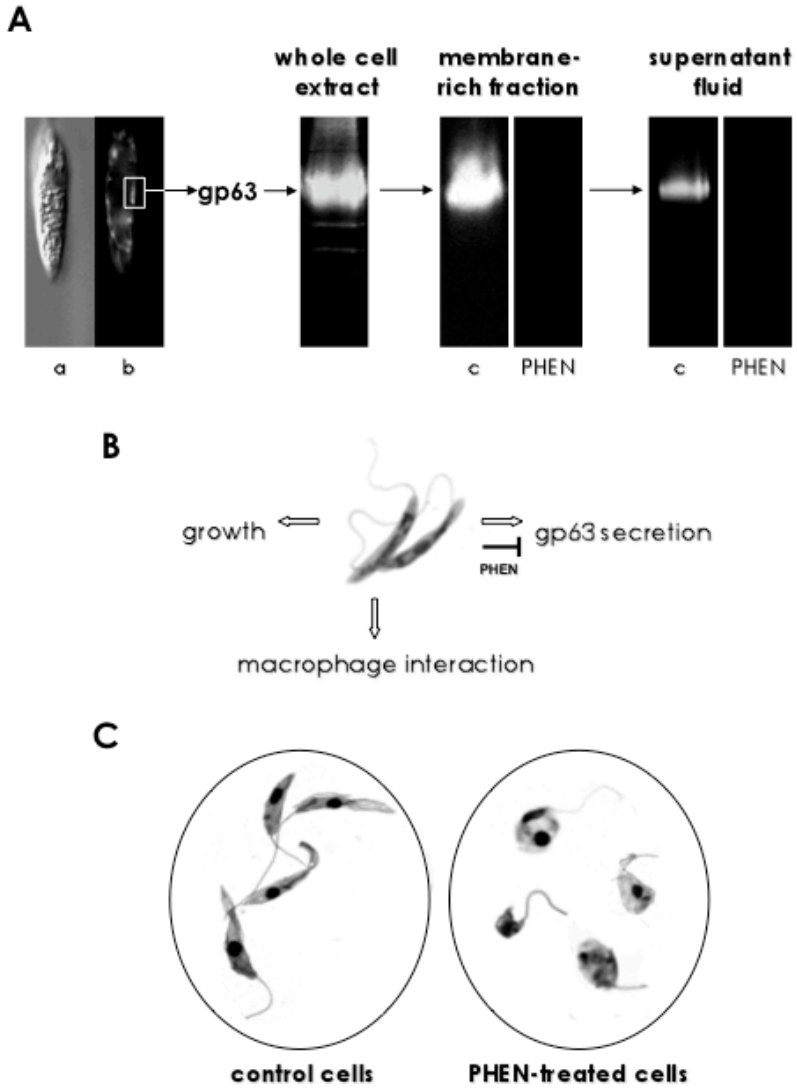

Fig. (4). Detection of the major metalloprotease (gp63) in *Leishmania braziliensis* and effects of PHEN on the parasite development. (**A**) Optical (a) and immune fluorescence (b) microscopies evidencing the surface location of the gp63 molecules after probing the promastigotes with an anti-gp63 antiserum. In sequence, gelatin-SDS-PAGE showing the alkaline protease profiles observed in the whole parasite extract obtained after lisys with SDS, in the membrane-rich fraction obtained after Triton X-114 extraction and in the cell-free culture supernatant. The gel strips were incubated in the absence (c) or presence of PHEN. (**B**) The treatment of *L. braziliensis* promastigotes with PHEN resulted in arrested growth, in the reduction of the gp63 secretion and in the blockage of the interaction with macrophages. Note: white arrows indicate that untreated parasites display normal growth, secrete molecules (including gp63) into the extracellular environment as well as interact with macrophages; however, black lines (PHEN) indicate the blockage of these crucial processes. (**C**) Morphological alterations were clearly observed after the treatment of promastigotes with 1 mM PHEN including parasites becoming short and round, which indicates a possible osmotic stress caused by the chelating agent. For experimental details see Lima and co-workers [99] and Cuervo and co-workers [285].

Results presented by Seay and co-workers [97], McGwire and co-workers [98] and Lima and co-workers [99] suggested that zinc-dependent metalloproteases participate in several crucial processes of *Leishmania* spp. life cycle. The best characterized *Leishmania* metalloprotease is a zinc-dependent glycoprotein of 60-66 kDa, named leishmanolysin or gp63, and which is abundantly expressed on the metacyclic promastigote surface (Fig. **4A**) and is involved in several steps of parasite-host interaction and virulence, including a protective role against the parasite degradation within macrophage phagolysosomes [93]. The spontaneous release of cell surface gp63 from metacyclic promastigotes was significantly reduced when the proteolytic activity of the protein was inactivated by site-specific mutagenesis or inhibited by zinc chelation with 1,10-phenanthroline (Fig. **4B**), suggesting that release involves autoproteolysis. The finding of a proteolytically active, extracellular gp63 produced by multiple *Leishmania* isolates suggests a potential role of the extracellular enzyme in substrate degradation relevant to their survival in both the mammalian host and the insect vector [98].

In *L. amazonensis*, the *in situ* inhibition of gp63 proteolytic activity inside *Leishmania*-infected macrophage phagolysosomes with targeted delivery of 1,10-phenanthroline selectively eliminated intracellular *Leishmania* amastigotes, suggesting the importance of this protease in the phagolysosomal survival of the parasite [97]. That inhibition of gp63 activity is the mode of killing of targeted 1,10-phenanthroline was further substantiated by the destruction of gp63-coated avirulent cells by 1,10-phenanthroline. This inhibition was not due to the non-specific chemical interaction of 1,10-phenanthroline with some other molecules but probably to its ability to chelate zinc, as shown by the ability of this metal to reverse the inhibitory effect of 1,10-phenanthroline.

As it is known that many virulence factors may have their expression decreased over several passages of parasites *in vitro*

[100], Lima and co-workers [99] confirmed that gp63 was mainly produced by *L. braziliensis* virulent promastigotes in comparison with avirulent promastigotes, and this proteolytic activity was drastically reduced after several *in vitro* passages of freshly isolated parasites from hamster lesions. 1,10-phenanthrolineand ethylene-glycoltetraacetic acid (EGTA) arrested the growth of a *L. braziliensis* virulent strain in a dose-dependent manner. Also, thepre-treated of promastigotes with 1,10-phenanthroline promoted a significant reduction in the interaction index with mouse peritoneal macrophages (Fig. **4B**). The promastigote growth was decreased even at the lowest concentration used (0.1 μM). Morphological alterations were detected in the virulent strain, including parasites becoming short and round (Fig. **4C**). Conversely, these chelating agents did not affect either the proliferation, cell morphology or the cellular interaction of the avirulent, laboratory-adapted strain. Moreover, the spent culture medium from the virulent strain significantly enhanced the association index, in a dose-dependent manner, between avirulent strain and macrophages, and this effect was reversed by 1,10-phenanthroline.

The inhibitory mechanism of 1,10-phenanthroline against metalloproteases is also seen against gp63 homologues detected in distinct trypanosomatids. For instance, one of the *T. bruceigp63* gene families is involved in the proteolysis process related to release of variant surface glycoprotein from the surface of transgenic procyclic parasites and this mechanism was inhibited by different zinc chelator compounds, including 1,10-phenanthroline [101, 102]. Homologues of the gp63 metalloprotease participate in relevant steps of the interaction of the insect trypanosomatid *Herpetomonas samuelpessoai* with murine macrophage cells, based on the reduction of the association index caused by 1,10-phenanthroline [103]. As similarly demonstrated in *Leishmania* species, 1,10-phenanthroline-treated *H. samuelpessoai* cells showed a considerable reduction in the release of surface gp63-like molecules to the extracellular medium [104], suggesting a similar mechanism of secretion in this trypanosomatid. In addition, Pereira and co-workers [105] demonstrated an augmentation in the secretion of gp63-like molecules (around 2-fold) by *H. samuelpessoai* cells during the contact with explanted guts of *Aedes aegypti* in comparison to parasites in axenic cultures, suggesting the possible participation of secreted gp63 molecules in the interaction process with invertebrate host. In contrast, *H. samuelpessoai* cells pre-treated with 1,10-phenanthroline and then allowed to interact with guts did not release detectable gp63-like molecules and presented considerable reduction by approximately 60% in the association index [105]. Furthermore, *H. samuelpessoai* differentiation (transformation of promastigotes into paramastigotes) was decreased in the presence of 1,10-phenanthroline [106]. *Phytomonas serpens*, a tomato trypanosomatid, also presents gp63 homologues [107]. EDTA, EGTA and 1,10-phenanthroline were able to arrest the growth of *P. serpens* with a distinct pattern of inhibition, with 1,10-phenanthroline being the most effective compound [107].

2.3. Calcium Chelators

In a series of studies, Mbati and co-workers [108] verified the effects of EGTA and EDTA against *L. donovani*. When promastigotes were treated with various concentrations of EDTA for 7 days, concentrations of up to 1 mg/ml produced no significant reduction in the promastigote population, while concentrations between 0.05 and 0.1 mg/ml did not significantly affect the viability of BALB/c mouse peritoneal macrophages. *L. donovani* parasites engulfed in macrophages were exposed to various concentrations of EDTA within the acceptable toxic levels for macrophages, with highest rates of parasite clearance at 0.1 mg/ml. The length of time (in days) which infected macrophages were treated contributed significantly to a decline in the level of parasite loads. Levels of calcium, magnesium and iron were virtually unchanged in the supernatant of cell cultures, while manganese levels were higher in EDTA-treated cultures as compared to the control [108].

Exposure of *L. donovani* promastigotes to EGTA concentrations from 0.2 to 1.6 mg/ml significantly inhibited their growth, while EGTA concentrations of 0.05 and 0.1 mg/ml were non-toxic to mouse macrophages *in vitro* [109]. In concentrations of 0.05, 0.1 and 0.2 mg/ml, EGTA contributed significantly to a decline in *L. donovani* amastigote loads within macrophages. However, when *L. donovani*-infected Syrian hamsters were treated intraperitoneally with 0.23 mM/kg/day of EGTA, EDTA, HEEDTA (hydroxy-2-ethylenediaminetriacetic acid) and 100 mg/kg/day of Pentostam R for 30 days, 5 out of 6 Pentostam-treated animals had negative spleen cultures, but all the chelator-treated animals yielded parasites. While all the Pentostam-treated animals had negative bone marrow cultures, only 1 out of 6 HEEDTA-treated hamsters yielded parasites [110]. Spleen, liver and bone marrow parasite loads calculated from chelator-treated animals were consistently higher than for Pentostam-treated animals, which suggest that although metal ion chelators had some antileishmanial potential, the *in vivo* activity against *L. donovani* was low compared to Pentostam.

The effects of EGTA treatment was also determined in *T. brucei* bloodstream forms incubated in a calcium-free medium containing 10 μM EGTA and the calcium ionophore, A23187 [111]. Under these conditions, calcium depletion led to the polymerization of additional microtubules in an extension of the endoplasmic reticulum at the flagellar attachment site as well as to a retraction of the endoplasmic reticulum extension from its usual position and nucleolus segregation. These data suggested that intracellular calcium regulation might be important for specific depolymerization/polymerization reactions during the course of cell division and the formation of functional ribosomes in *T. brucei*.

The effects of the co-administration of EDTA and diminazene aceturate (DA), the most widely used drug against nagana cattle disease, was investigated on the level of parasitemia, parasite clearance, packed cell volume (PCV) and post-infection survival time (PIST) in albino mice infected with DA-resistant *T. brucei* [112]. The co-administration of EDTA and DA led to a slight potentiation of DA, as seen by a decreased parasitemia, improvements in PCV and a marginally higher PIST when compared to DA-treated mice. The administration of EDTA alone significantly enhanced the resistance of the infected mice, since EDTA-treated, infected mice presented a significantly lower level of parasitemia, improved PCV and higher PIST than infected, non-treated mice.

In order to study the possible role of the intracellular calcium level in the regulation of *T. cruzi* infectivity, the capacity of trypomastigote forms of this organism to invade mammalian cells was measured after treatments which decrease or elevate cytoplasmic calcium levels [113, 114]. In parasites loaded with either bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′ tetraacetic acid (BAPTA) or 2-{[2-bis(carboxymethyl)-amino-5-methylphenoxy] methyl}-6-nethoxy-8-bis(carboxymethyl)aminoquinoline (Quin-2) at 50 μM to chelate calcium, a decrease in both the proportion of rat heart myoblasts invaded by the parasite *in vitro* and the number of trypanosomes penetrating these host cells was observed, in a process that was dose-dependent. Consistent with these findings, parasites pretreated with the calmodulin-binding phenothiazines, trifluoperazine and chlorpromazine, or with felodipine, a chemically different type of calmodulin antagonist, also presented decreased infectivity. In contrast, pretreatment with the calcium ionophore, ionomycin, which elevated calcium levels in *T. cruzi*, significantly enhanced the infective capacity of the parasite, which pointed to the existence of a calcium-dependent mechanism that

regulates the invasive capacity of *T. cruzi*, as reviewed by Yoshida and Cortez [115].

3. ANTI-*PLASMODIUM* ACTION OF CHELATING AGENTS

More than 125 species of *Plasmodium* are able to infect reptiles, birds and mammals. Usually, four of them are able to infect human and cause malaria: *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax* and *Plasmodium ovale* [116]. In 1965, it was described the first human infection by *Plasmodium knowlesi*. From that time on, others cases were reported [117-120]. Known since antiquity, malaria still remains one of the main hazards to human health. Half of the world's population is at risk of malaria and likely one million deaths occur due to the disease each year. The goal of malaria control is to reduce the morbidity of and mortality from the disease [121]. In this context, antimalarial medicines are one of the great tools in malaria control by ending patient malaria infection, thus contributing to the reduction of the transmission of the disease. During the past years, there has been a wide occurrence of drug-resistant parasites in many areas where malaria occurs [122]. *P. falciparum* strains resistant to chloroquine [123, 124], a drug allowed almost a century for malaria control, was first reported in Malaya in 1962. It did not take long for most of the strains of *P. falciparum* became resistant to quinolones and others medicines such as sulfadoxine-pyrimethamine. It was also reported, in some countries, resistance of *P. vivax* to chloroquine [125-129]. This scenario has led to the increasing of efforts to search for new effective antimalarial therapies. In the 1980s two classes of antimalarials were discovered: endoperoxides and iron chelators [130]. The antimalarial action of iron chelators is based on three factors: their capacity in binding iron(III), penetrating much faster into parasitized erythrocytes and egress from parasites after treatment [131]. Clinical studies showed that the iron supplementation to iron-deficient individuals causes exacerbation of malaria [132]. Unlike mammalian cells that obtain their iron primarily from transferrin [133], parasites mobilize iron from the infected red blood cells [134-136].

DFO has been shown to suppress malaria parasite growth both *in vitro* [134, 135, 137] and *in vivo* [138, 139]. DFO diminished the parasitemia caused by *P. falciparum* and *P. vivax* in humans, but recrudescence occurred after the initial clearance [140, 141]. Cerebral malaria was prevented by DFO in mice infected with *P. berghei* [142], but in human cerebral malaria therapy, its effect was not clear. It has been reported that DFO may quickness the clearance of parasitemia and increase recovery from deep coma in children with cerebral malaria [138]. However, drug efficacy is dependent on many factors like immune modulation responses [144]. In this context, a combination of quinine with DFO increased the serum concentrations of the stable end products of nitric oxide (NO), nitrite and nitrate in Zambian children with cerebral malaria compared with those group given placebo and quinine. In addition, IL-6 and IL-10 levels were raised initially and decreased considerably in both groups over 3 days. Accordingly, DFO could support the Th1-mediated immune effector function involving increased production of NO in children with cerebral malaria [145].

DFO is markedly slower in permeating membranes [146, 147] and affects primarily trophozoite/schizont stages [148]. *In vitro*, DFO is known to be active against *P. falciparum* with IC_{50}s extending from 10 to 30 μM [149-152] in rodents [153] and in humans [154, 155]. Due to its short half-life in plasma (5-10 min) and poor absorption following oral administration, DFO application in malaria therapy is very limited [156]. So, two subfamilies of lypophilic iron(III) chelators, capable in penetrating easily in the membrane of red blood cells were designed [157, 158]. These compounds are hydroxamate-based, similar to DFO, named reversed siderophores [157]. $RSFileu_{m2}$, a reversed siderophore, affected the development of parasite and mammalian cells by chelating intracellular iron(III) [159]. Although cellular energy production depends on iron-containing enzymes, both $RSFileu_{m2}$ and DFO promoted only 30% reduction of ATP synthesis in malaria parasites in infected red blood cells. In the ring stage, DNA synthesis was irreversibly inhibited by $RSFileu_{m2}$ in a great scale compared to that observed by DFO, but during trophozoite stage the inhibition effect was greater with DFO [148]. This was probably due to the different permeation properties at these two development stages of the parasite and the retention of the drug inside parasites after its removal [160].

Several other classes of iron(III) chelators have been under consideration for malaria chemotherapy. The N^4-nonyl,N^1,N^8-bis(2,3-dihydroxybenzoyl) spermidine hydrobromide, FR160 (R = C_9H_{19}), was the first iron-chelating compound developed particularly for antimalarial action [161]. It is a catecholate siderophore derived from spermidine that affected the parasites at considerably faster rates and at all stages of parasite growth [162]. FR160 is 14-fold more potent than DFO, and also more potent than the majority of the reversed siderophores tested *in vitro*, with IC_{50}s ranging from 3 to >100 μM [163-165]. FR160 was most effective in trophozoite and young schizont stages, but also affected rings and schizonts. The mode of action of FR160 seems to be bygenerating radical species and improvement of heme-catalyzed oxidation of lipid membranes, different from those used as antimalarial drugs. Additionally, the ribonucleotide reductase of *P. falciparum*, a key iron-dependent enzyme in pyrimidine *de novo* synthesis, could be a target for FR160, as for DFO[161]. Pradines and co-workers [165] showed the synergic effects of FR126 with doxycycline or atovaquone that favors the use of both in combination for increasing the antimalarial activities. Besides, the effects of FR160 on mammalian cells in culture were minimal compared with those obtained with human malaria parasites [164].

Like iron, copper is an essential element for organisms in general. It acts as a cofactor of various enzymes and as a reduction-oxidation reactive metal, which can generate toxic free ionic copper that is sequestered by particular mechanisms [166, 167]. Like iron chelators, several copper chelators have antimalarial properties *in vitro* [167-169]. Extracellular chelation of copper by 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulphonic acid disodium salt (BCS) did not inhibit parasite growth, indicating that *Plasmodium* did not need to obtain copper from serum, but can rely on erythrocytes sources [167]. Cu, Zn superoxide dismutase is supposed to be a source of copper to the *Plasmodium* parasite because it is the most abundant copper-containing enzyme in erythrocytes. It has been demonstrated that copper sulfate at 5 μM inhibits more than 50% of parasite growth, which is overturned by 200 μM diethylenetriaminepenta-acetic acid, an impermeant metal chelator [170]. An intracellular copper chelator, neocuproine (2,9-dimethyl-1,10-phenanthroline), prevented parasite growth specifically at ring-trophozoite stage transition and reduced the intraparasitic levels of both Cu and Zn superoxide dismutase and catalase [167].

4. ANTIFUNGAL ACTION OF CHELATING AGENTS

Fungal infections are an increasing cause of morbidity and mortality in hospitalized patients in the last three decades, especially in immunocompromised individuals [171]. Many fungi can cause invasive diseases, with *Aspergillus* spp. and *Candida* spp. being the most prevalent fungal pathogens infecting susceptible patients. During the past few years, rare molds like zygomycetes (*Mucor* spp. and *Rhizopus* spp.),*Fusarium* spp., *Scedosporium* spp. and many other less frequent fungal pathogens have come into focus as the cause of devastating clinical diseases[172, 173]. The

management of invasive fungal disease in immunocompromised hosts is complex due to several aspects, such as the limitation of potent and non-toxic antifungal drugs [174].

4.1. Iron Chelators

Iron acquisition is essential for the growth and virulence of *Aspergillus*spp. [175]. Iron chelators were able to modulate *Aspergillus fumigatus* conidial growth behavior in different ways: lactoferrin (IC_{50} = 105 ± 9 nM), ciclopirox (IC_{50} = 4.22 ± 0.18 μM) and deferiprone (IC_{50} = 1.29 ± 0.2 mM) significantly inhibited, while DFO enhanced fungal growth. By means of checkerboard assays, antifungal synergy against conidia was observed for combinations of ketoconazole with ciclopirox or deferiprone, lactoferrin with amphotericin B, and fluconazole with deferiprone [176].

Transfusional siderosis and iron overload in malignancy and liver and stem cell transplantation are associated with an increased risk of aspergillosis [177-179]. Ibrahim and co-workers [180] reported that deferasirox, the first orally available iron chelator approved by the FDA, with an indication for the treatment of transfusion-related iron overload [181],had a modest but significant effect against *A. fumigatusin vitro*, presenting cidal action and a minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 25 and 50 mg/L, respectively. Deferasirox monotherapy modestly prolonged survival of mice with invasive pulmonary aspergillosis, but a combination of deferasirox (at 10 mg/kg twice a day) and liposomal amphotericin B (LAmB, at 3 mg/kg/day) therapy synergistically improved survival and reduced lung fungal burden compared with either monotherapy alone [180]. Holbein and Mira de Orduña [182] reported the influence of the exogenous chelating agents, lactoferrin, deferiprone, DFO, bathophenanthroline sulphonate and a novel chelator with a hydroxypyridinone-like Fe-ligand functionality (named DIBI) on *Candida albicans* growth. The results demonstrated that *C. albicans* was resistant to lactoferrin at physiologically relevant concentrations and DFO, but was inhibited by low concentrations (0.25 g/l) of DIBI for 24 h and displayed very weak growth thereafter. Recent studies of the role of iron in candidiasis have revealed that this element is important not only for the normal function of host immunity but also for pathogenic *Candida* owing to the fact that absence of this metal resulted in reduced virulence and hence reduced yeast invasion of the host epithelium [183]. In addition, iron deprivation is a mechanism by which drug susceptibility is enhanced in *Candida* cells by increasing the membrane fluidity (lowering ergosterol content), which in turn leads to enhanced passive diffusion of drugs [184].It is well known that *C. albicans* causes endothelial cell injury both *in vitro* and *in vivo*. In parallel, iron is critical for the induction of injury in many types of host cells. With this task in mind, Fratti and co-workers [185] found that the pretreatment of endothelial cells with DFO protected them from candidal injury, even though the organisms germinated and grew normally. The cytoprotective effects of iron chelation were likely due to the reduced phagocytosis of this fungus by endothelial cells [185].Ciclopirox inhibited the growth of *C. albicans* yeasts as well as their transformation into germ tubes, in a dose-dependent fashion [186]. The treatment with ciclopirox did not cause cell membrane damage or change in intracellular levels of ATP. Conversely, ciclopirox induced intracellular glucose-6-phosphatedehydrogenase, reduced catalase and did not influence superoxide dismutase activity. Finally, the transcriptional profiling pattern of drug-treated *C. albicans* cells strongly resembled iron-limited conditions [186].

The growth of *Cryptococcus neoformans* under solid minimum medium containing low amount of iron (<0.5 μM) was inhibited by 100-200 μg/ml (0.28-0.56 mM) of the metal-free iron chelator ethylenediamine-di(o-hydroxyphenylacetic acid) (EDDA) [187]. This medium supported good growth at pH 6.0 but poor growth at pH 7.5, presumably because iron(III) ions are less soluble at neutral pH [187]. Similarly, the iron(II) ion chromophore bathophenanthroline sulphonate (BPDS) inhibited the growth of *C. neoformans* in iron-limited media [188]. The yeasts also acquired iron as [$^{59}Fe^{3+}$]-citrate and [$^{59}Fe^{3+}$]-pyrophosphate, while BPDS reduced the acquisition of iron(III) ions by these ligands around 50% [189]. Ciclopirox showed fungistatic activity against *C. neoformans* in concentrations ranging from 0.25 to 1 μg/ml and fungicidal activity in concentrations from 0.5 to 4 μg/ml [190]. Agh-Atabay and co-workers [191] described the synthesis of a series of bisbenzimidazole-derived chelating agents which displayed effective action against *C. neoformans*. These ligands included 1,2-bis(2-benzimidazyl)-1,2-ethanediol; 1,4-bis(2-benzimidazyl)-1,2,3,4-butanetetraol; 1,3-bis(2-benzimidazyl)-2-thiapropane; 1,3-bis(2-benzimidazyl)-2-thia-propane-dihydrochloride; 1,5-bis(2-benzimidazyl)-3-thiapentane and 1,5-bis(2-benzimidazyl)-3-thiapentane dihydrochloride.

DFO, at concentrations of 0.5 and 1 mM, inhibited the *in vitro* growth of *Histoplasma capsulatum* yeast cells. However, 0.1 mMDFO had only a slight inhibitory effect [192]. In addition, the treatment of mouse peritoneal macrophages with DFO (0.1-1 mM) inhibited the intracellular growth of the yeasts in a dose-dependent manner, suggesting that the intracellular development of *H. capsulatum* is dependent on iron [192]. Newman and co-workers [193] reported that the iron chelator, VUF 8514, a 2,2'-bipyridyl analog, effectively inhibited the growth of *H. capsulatum* yeasts in both culture medium (IC_{50} = 30 nM) and within human macrophages (IC_{50} = 520 mM).

Clinical and animal data indicate that the presence of elevated available serum iron (like in diabetic ketoacidosis and other systemic acidosis) predisposes the host to mucormycosis by inducing dissociation of iron from sequestering proteins, resulting in elevated available serum iron [194-196]. In addition, patients treated with DFO have a markedly increased incidence of invasive mucormycosis, which is associated with a mortality of >80% [197]. For example, DFO predisposes patients to *Rhizopus* infection by acting as a siderophore, which supplies previously unavailable iron to the fungus [198]. *Rhizopus* obtains iron from the iron-DFO complex by intracellular transport of the reduced iron without DFO internalization [199]. On the other hand, Ibrahim and co-workers [200, 201] reported that deferasirox induced an effective iron-starvation response in *Rhizopus oryzae*. Also, deferasirox showed fungicidal activity *in vitro* against 28 of 29 (97%) clinical isolates of Mucorales (*Mucor* spp., non-*oryzae Rhizopus* spp. and *R. oryzae*) at concentrations ($MICs_{90}$ = 3.12-6.25 μg/ml) well below clinically achievable serum levels. The *in vivo* efficacy of deferasirox was tested in diabetic ketoacidotic mice infected with spores of *R. oryzae*, mimicking a disseminated model of murcomicosis. When administered orally twice daily for 7 days (starting the day after infection), deferasirox at 1, 3 or 10 mg/kg significantly improved survival, in which a dose-response trend was observed, with more surviving animals in higher-dose groups, as well as decreased tissue fungal burden, with an efficacy similar to that of liposomal amphotericin B [200]. In addition, deferasirox was able to cure mice infected intranasally with *R. oryzae*, showing its efficacy to control pulmonary mucormycosis. Deferasirox treatment promoted an enhancement in the host inflammatory response to mucormycosis, with high levels of IFN-γ. Most importantly, deferasirox synergistically improved survival and reduced tissue fungal burden when combined with liposomal amphotericin B [200]. Corroborating these findings, Chamilos and co-workers [202] demonstrated that using *Drosophila melanogaster* as a model host, deferasirox significantly protected wild-type flies infected with *R. oryzae* when compared with placebo-treated flies. Interestingly, deferasirox was successfully used as a salvage therapy to treat a patient with rhinocerebral mucormycosis, who

was failing months of polyene treatment [203].Deferiprone (1,2 dimethyl-3-hydroxy-4(1H)-pyridinone, also known as L1, CP20, Ferriprox or Kelfer) is a member of the α-ketohydroxypyridine class of iron chelators and is approved for use in iron overload conditions in India and Europe [204, 205], but is not approved by the USA FDA for use in humans. Deferiprone demonstrated static activity against *R. oryzae* at 24 h, but showed fungicidal activity at 48 h of incubation. Deferiprone was as effective as LAmB at improving survival and decreasing brain fungal burden, and both drugs were more effective than placebo in non-iron-overloaded animals [206].

DFO strongly inhibited the *in vitro* growth of *Penicillium marneffei* in a dose-dependent manner. As expected, this inhibition was reversed by adding an inorganic ($FeCl_3$) or an organic (hemin) iron source [207].DFO also significantly reduced the lung fungal counts in a rat model of acute *Pneumocystis carinii* pneumonia, in a dose-dependent manner, when it is given either as a daily bolus dosage or by continuous infusion [208-212]. Similarly, Weinberg and Shaw [213] showed that DFO suppressed the growth of *P. carinii* in an established human embryonic lung fibroblast cell lineage at concentrations safely achievable in human serum (5-15 μg/ml, corresponding to 7.6-22.8 μM). In this same line, Weinberg [214] described that the hydroxypyridinone iron chelators, which are administered orally to humans, showed the ability to inhibit the *in vitro* growth of *P. carinii*, which was correlated with alterations in *P. carinii* morphology, as viewed by transmission electron microscopy. In an elegant work conducted by Clarkson and co-workers [215], it was demonstrated that DFO was able to penetrate *P. carinii*, causing irreversible cell damage, thus indicating a different mode of action. This chelating agent also caused a reduction in *P. carinii* cytoplasmic free iron. Furthermore, the authors reported the complete efficacy of weekly aerosol delivery of DFO to the lungs of *P. carinii*-infected rats using an experimental design intended to deliver an amount of DFO to the lungs equivalent to that delivered by an intraperitoneal injection of 1000 mg/kg, a dose that had previously been shown to be effective if given every day for 21 days [210, 215]. The results revealed that the once-a-week aerosol treatment of rats was 100% effective both as a prophylactic and as a curative treatment in a rat model of *P. carinii* pneumonia [215]. Daphnetin (7,8-dihydroxycoumarin), a known iron chelator, demonstrated to exhibit *in vitro* activity against *P. carinii* in a short-term axenic culture system [216]. The compound was found to suppress the growth of *P. carinii* in a dose-dependent manner at concentrations between 1 and 20 μM. As determined by transmission electron microscopy, the reduction of *P. carinii* numbers after treatment with daphnetin correlated with morphological changes in the fungal cells.

4.2. Zinc and Calcium Chelators

Autophagy is the major cellular pathway for bulk degradation of cytosolic material and is required to maintain viability under starvation conditions [217]. The disruption of the *A. fumigatus atg1* gene, which encodes a serine/threonine kinase required for autophagy, showed abnormal conidiophore development and reduced condition, although the defect could be bypassed by increasing the nitrogen content of the medium [218]. EDTA induced autophagy in *A. fumigatus* even in the presence of abundant carbon and nitrogen, and the Δ*Afatg1* mutant was severely growth-impaired under these conditions. The stimulation of autophagy by EDTA suggested that autophagy has a role in protecting the organism from the deleterious consequences of divalent cation depletion [218]. These data establish a role for autophagy in starvation-associated foraging and provide an unanticipated link between autophagy and metal ion homeostasis [219].

Analysis of the combined effects of different drugs is clinically important since synergistic combinations of drugs may be more efficacious and less toxic than single agents [220, 221]. With this task in mind, Hachem and co-workers [222] described that EDTA is an adjunct antifungal agent for invasive pulmonary aspergillosis in a rodent model. Immunosuppressed rats were infected with *A. fumigatus* and then treated with amphotericin B lipid complex (5 mg/kg of body weight/day for 7 days), EDTA (30 mg/kg/day for 7 days) or a combination of both compounds. The mortality rate was reduced, the duration of survival was increased, fewer *A. fumigatus* organisms were recovered from the lungs, and less-severe lung lesions and minimal angioinvasion were seen histopathologically in the rats receiving the combination treatment than in the rats receiving either the antifungal agent or EDTA alone. These findings suggest that the two drugs have an additive effect and that concurrent therapy with EDTA and amphotericin B lipid complex may be of value in patients with hematologic malignancies, especially in patients with persistent neutropenia. All of the tested animals showed normal levels of both serum calcium and creatinine at different times during the therapy. Furthermore, no tissue damage was seen in the histopathologic specimens that could be attributed to the EDTA. It was concluded that EDTA potentiates amphotericin B lipid complex without additional toxicity [222].

Several well-known virulence factors produced by *Aspergillus* spp. are inhibited by chelating agents, justifying their potent antifungal action. For example, (i) an immunogenic 40 kDa secreted metalloprotease (named MEP) of *A. fumigatus*, which is able to cleave collagen, was totally inhibited by 1 mM EDTA and 1 mM 1,10-phenanthroline [223]; (ii) an elastinolytic neutral metalloprotease of 43 kDa, which was expressed during the invasion of neutropenic mouse lungs, was fully inhibited by EDTA and 1,10-phenanthroline (at 1 mM) [224]; (iii) a 33 kDa serine protease of *A. fumigatus* with elevated elastastinolytic activity is also inhibited by 5 mM EDTA (100%) and 10 mM 1,10-phenanthroline (30%), suggesting a requirement for divalent cations [225, 226]; (iv) a nuclease composed of three subunits of 80 kDa, 50 kDa and 25 kDa of *A. sydowii* was inactivated by EDTA and EGTA [227]; (v) Cu,Zn superoxide dismutases from *A. fumigatus*, *A. flavus*, *A. niger*, *A. nidulans* and *A. terreus* were inhibited by 1,10-phenanthroline and EDTA at concentrations from 5 to 30 mM [228]; (vi) EDTA at 50 mM inhibited the binding (30%) of live conidia of *A. fumigatus* to human Langerhans cells via a lectin of galactomannan specificity [229].

Several published works have demonstrated that the metal-free 1,10-phenanthroline and a number of transition metal complexes incorporating this chelating ligand were extremely active, *in vitro* at 37◦C, against *C. albicans*. For instance, metal-free 1,10-phenanthroline and the copper(II) and manganese(II) phenanthroline complexes (general formulae: $[M(phen)_2(H_2O)_n]^{2+}$; M = Cu or Mn) were potent growth inhibitors of *C. albicans* at 20 μg/ml [230]. Also, complexes which were active against *C. albicans* also proved effective against *C. glabrata*, *C. tropicalis* and *C. krusei* with the manganese complexes retaining superior activity [230]. The antifungal properties and the mechanisms of action of phenanthroline derivatives have been investigated. In this sense, the potent copper(II) complex, $[Cu(phen)_2(mal)].2H_2O$; ($malH_2$ = malonic acid), induced significant cellular oxidative stress, decreased reduced:oxidized glutathione ratios (GSH:GSSG), increased levels of lipid peroxides [231], caused damage mitochondrial function (reducing respiratory function and the levels of cytochromes b and c in the cells) and reduced the levels of ergosterol [232]. Exposure of *C. albicans* to $[Mn(phen)_2(mal)].2H_2O$ or $[Ag_2(phen)_3(mal)].2H_2O$ resulted in DNA degradation, whereas exposure to 1,10-phenanthroline or $[Cu(phen)_2(mal)].2H_2O$ did not. An inspection by transmission electron microscopy revealed that all these compounds induced extensive changes to the internal structure of yeast cells including retraction of the cytoplasm, nuclear fragmentation and disruption of the mitochondrion [233]. Using the insect *Galleria mellonella* as a

model to test the antifungal efficacy of phenanthroline derivatives, Rowan and co-workers [234] reported that insect larvae pre-inoculated with either [Ag_2(mal)(phen)$_3$]) or 1,10-phenanthroline were protected from a subsequent lethal infection by *C. albicans*, while larvae inoculated 1 and 4 h post-infection showed significantly increased survival in comparison to control larvae [234]. In addition, several metallo-type enzymes produced by *C. albicans* are targets for 1,10-phenanhroline inhibition, including different zinc-metallo-type proteases [235-238].

Two particular ways for EDTA to show its antifungal activities are anti-colonization and anti-growth properties. In this sense, Odds [239] suggested that there is a direct relation between adherence of *C. albicans* and its ability to colonize and cause disease. The presence of calcium ions has been shown to have a critical role in the control of morphogenesis [240] and the adherence capacity of *C. albicans* to various extracellular matrix proteins [241]. By chelating calcium ions in the medium, EDTA prevents binding of *C. albicans* to these proteins in a dose-dependent manner. In the second process, EDTA reduces the growth of *C. albicans* by removing calcium ions from the cell walls and causing collapses in the cell wall as well as inhibiting enzymatic reactions [242]. Treatment with EDTA, EGTA and 1,10-phenanthroline also reduced the cell viability of *C. parapsilosis*, in a dose-dependent manner, altering its membrane permeability to propidium iodide (Fig. **5A**).

1,10-phenanthroline completely inhibited the transformation of 24- to 48-hour stationary phase singlet cells of *C. albicans* into mycelia as well as restricting the bud formation (around 10%) at minimal concentrations of 50 μM and 230 μM, respectively. As expected, the inhibition profiles of both phenotypes could be reversed completely by the addition of 200 μM of $ZnSO_4$ [243]. The yeast into hyphal development in *C. albicans* was blocked by EDTA and this effect was not due to a general growth inhibition, since the chelator did not affect either protein or DNA synthesis. Corroborating this finding, recovery of mycelial growth was observed when EDTA-grown cells were incubated in an EDTA-free medium. High-molecular-weight mannoproteins (HMWM) that are mycelium-specific wall components, and particularly a 260 kDa species (HMWM-260), were absent in the wall of cells grown under germination conditions in the presence of EDTA. Synthesis of the HMWM-260 species was not inhibited but its incorporation (secretion) into the wall structure seemed to be blocked in EDTA-treated cells [244]. The surface mannoproteins are directly linked to the adhesive properties of *C. albicans* [245]. In this sense, EDTA and EGTA modified the attachment of blastoconidia of *C. albicans* to platelets, suggesting a cation-dependent linkage. In contrast, the fixation of *C. glabrata* and *C. tropicalis* was not modified by chelating agents [246]. Similarly, the treatment of *C. parapsilosis* yeasts with either EDTA or EGTA or 1,10-phenanthroline did not perturb the interaction process with epithelial cells (HEp-2) (Fig. **5B**).

Candida albicans can readily form biofilms on both inanimate and biological surfaces. *Candida* biofilms have innate resistance and/or tolerance to hard surface disinfectants, metal ions and antifungal drugs in comparison to planktonic suspensions of fungal cells [247]. Corroborating this finding, the hyphal wall protein *HWP1* gene expression was reduced in EDTA-treated planktonic and biofilm samples. These results suggest that EDTA inhibits *C. albicans* biofilm formation most likely through its inhibitory effect on filamentation [248]. Yeast into germ tube differentiation was totally inhibited by EDTA even at the lowest concentration of 0.1 μM, while EGTA and 1,10-phenanthroline arrested this cellular transformation in a lower extension and in a typically concentration-dependent manner (Fig. **6A**). As expected, chelating agents also suppressed the biofilm formation in *C. albicans* (Fig. **6B**). Harrison and co-workers [249] reported that EDTA, at concentrations of ≥2 mM, killed approximately 90–99.5% of the

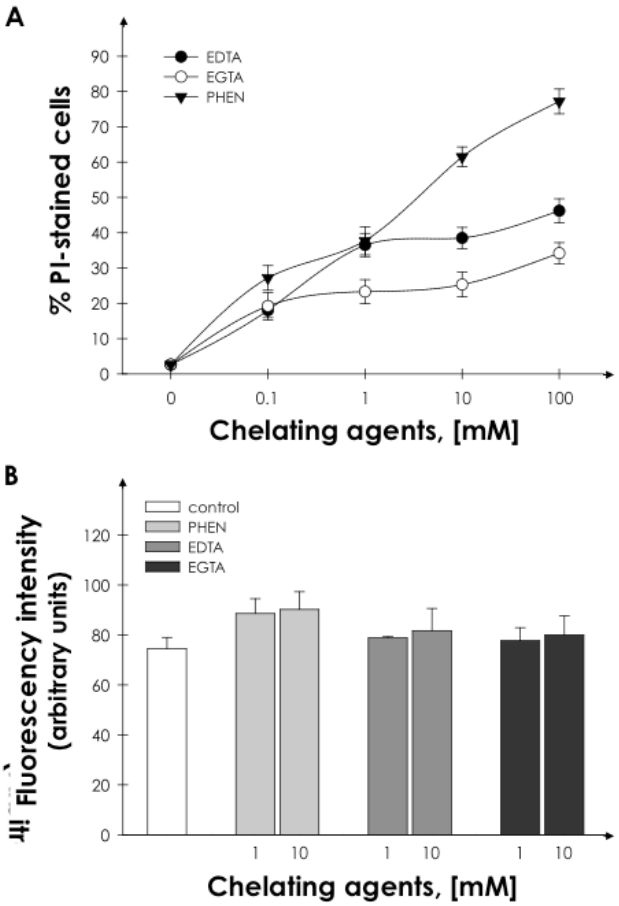

Fig. (5). Effect of chelating agents on the proliferation rate and on the interaction process with epithelial cell by *Candida parapsilosis*. (**A**) Yeasts (10^6) were incubated for 24 h in the absence or presence of different concentrations of PHEN, EDTA or EGTA. Fungal viability was measured by propidium iodide incorporation through flow cytometry analysis. (**B**) For the interaction assay, initially the *C. parapsilosis* yeasts (10^6) were incubated for 1 h in the presence of different concentrations of the chelating agents. After this treatment, the viability of the yeasts was not affected as judged by propidium iodide staining (data not shown). Subsequently, the yeasts were stained for 10 min with 0.5 mg/ml fluorescein isothiocyanate (FITC; Sigma) and then interacted for 2 h with epithelial cells (HEp-2 lineage) (10 fungi per epithelial cell). After removal from the plastic surface, the cells were analyzed by flow cytometry. For experimental details see Chaka and co-workers [286].

biofilm cell populations. Notably, a small fraction (around 0.5–10%) of biofilm cells was able to withstand the highest concentration (16 mM) of this chelating agent. Furthermore, *C. albicans* survivors from EDTA exposure that were propagated *in vitro* gave rise to biofilms with unchanged susceptibility to this agent. These data suggest that a subpopulation of *C. albicans* biofilm cells is innately recalcitrant to the fungicidal action of organic metal ion chelators [249]. The combination of different compounds presenting antifungal action can solve this question. In this regard, Raad and co-workers [250] demonstrated that the EDTA (30 mg/ml)-minocycline (0.1 mg/ml) combination acted synergistically against free-floating *C. albicans* and *C. parapsilosis* in a suspension. Similarly, a flush solution consisting of minocycline and EDTA was also highly efficacious in preventing catheter-related colonization by *C. albicans* and *C. parapsilosis* cells embedded in biofilm on both *in vitro* and *ex vivo* models of silicone catheter colonization [251-253]. Similarly, EDTA-acetylsalicylic acid (aspirin)combination promoted an effective

action against established biofilm of *C. albicans* [254]. Besides, EDTA enhanced the antifungal activity of amphotericin B lipid complex against *C. albicans* and *C. parapsilosis* embedded in biofilm [255].

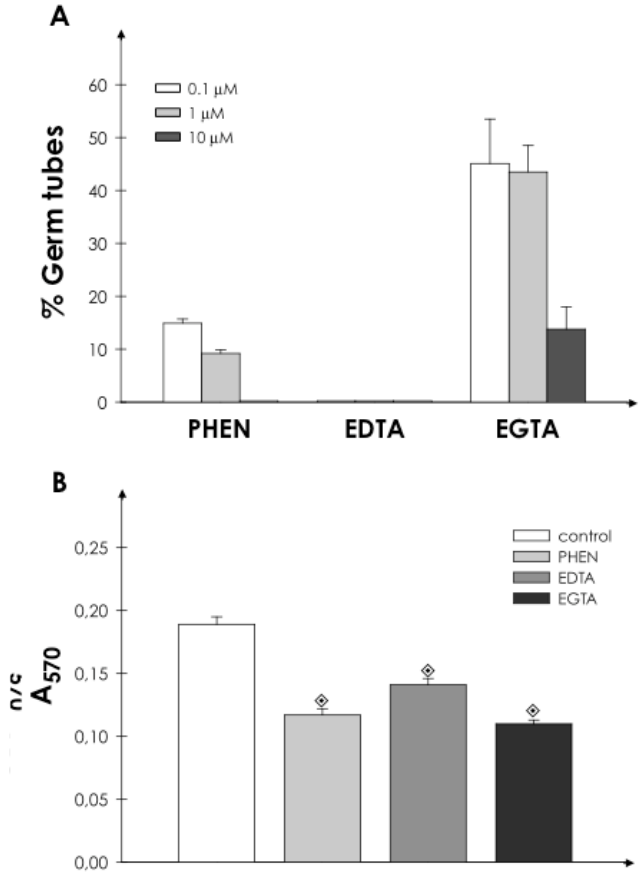

Fig. (6). Effect of chelating agents on the germination (yeast into germ tube transformation) and on the capability of biofilm formation by *Candida albicans*. (**A**) Germination was performed by incubating the yeasts in fetal bovine serum for 3 h at 37ºC in the presence of PHEN, EDTA and EGTA at different concentrations. The germination of fungal cells incubated in the absence of chelating agent was taken as 100%. For experimental details see Braga-Silva and co-workers [287]. (**B**) Yeasts were plated to adhere to the polystyrene substrate in the absence (control) or presence of chelating agents at 0.1 mM and then the systems were incubated for 48 h at 37ºC. Biofilm formation was quantified by crystal violet staining. *P* values of 0.05 or less were considered statistically significant in relation to the control (◈, Student's t-test). For experimental details see Braga-Silva and co-workers [288] and Braga-Silva and Santos [289].

Antimicrobial lock solutions may be needed to salvage indwelling catheters in patients requiring continuous intravenous therapy [252]. Several groups of investigators have demonstrated that EDTA solutions can be used to prevent catheter-related infections by clinically relevant microorganisms, including *C. albicans*. In this sense, Percival and co-workers [256] found that EDTA, at a concentration of 40 mg/ml, and applied for 21-25 h reduced the biofilm colonization of *C. albicans* on central venous catheter segments. EDTA also proved active against biofilms in catheters with significant reductions in biofilm viable counts after it was used to treat of hemodialysis catheters for 3 h [257]. In an interesting report, Devine and co-workers [258] showed that an EDTA solution efficiently disinfected toothbrushes and discs of polymethyl methacrylate (a denture base compound), eradicating biofilms derived from salivary inocula or pure cultures of *C. albicans*. In another study centered on irrigants for root canals, Sen and co-workers [259] evaluated the antifungal effect of EDTA on *C. albicans*, comparing it to various disinfectants and common antifungal agents. The authors reported that a 17% solution of EDTA showed the highest antifungal activity in comparison with routine antifungal drugs (nystatin and ketoconazole) and other solutions (sodium hypochlorite, chlorhexidine, hexetidine, benzalkonium chloride and povidone-iodine).

The anti-*C.albicans* activity of human salivary mucin MUC7 12-Mer-L peptides (which corresponds to amino acids 40 to 51 of the parent MUC7, the low-molecular-mass human salivary mucin) was enhanced by the addition of 1 mM EDTA to human clarified and unclarified saliva [260]. This was due to the chelation of divalent cations present in the saliva, which are known to inhibit the antifungal activity of the peptides. EDTA also improved the antifungal action of three other salivary antimicrobial peptides: MUC7 12-Mer-D (that contains all-D-amino-acid isomer), Hsn5 12-mer (corresponds to histatin 5) and magainin-II. In addition, EDTA is able to restrain the cell-associated collagenolytic activity produced by *C. albicans* [261].

Morphological transitions are fundamental steps during the infective process of dematiaceous fungi, such as *Exophiala dermatitidis*, *Cladophialophora carrionii*, *Phialophora verrucosa* and *Fonsecaea pedrosoi*. These are denominated as polymorphic fungi. *C. carrionii*, *P. verrucosa* and *F. pedrosoi*present a soil saprophytic phase, in which conidial and mycelial forms are found, and a parasitic stage, consisting of spherical, brownish-yellow cells with thick, deeply pigmented walls known as sclerotic cells/bodies[262], while the parasitic phase of *E. dermatitidis*is composed of budding yeasts [263]. The production of large numbers of sclerotic bodies occur when fungal cells were incubated at 37ºC in a chemically defined growth medium, at very low pH (around 2.5) and 0.1 mM Ca^{2+}ions [263, 264].In contrast, higher concentrations of Ca^{2+}ions reversed this tendency and promoted maintenance of hyphal growth. Addition of EGTA to the same medium, buffered at pH 6.5,also induced sclerotic bodies, but in a more concentration-dependent fashion: EGTA at 0.5-1.0 mM induced maximum numbers of sclerotic bodies in *C. carrionii*, while EGTA at 2 and 8 mM was required for the same results in *F. pedrosoi* and *P. verrucosa*, respectively [263, 264].Phenotypic switching in *E. dermatitidis* between polarized growth processes leading to yeast budding or hyphal apical extension and nonpolarized processes leading to isotropically enlarged forms that may become multicellular is a cellcycle-related phenomenon. In addition, EGTA at concentration ranging from 5 to 10 mM arrested isotropic cellular growth, mitosis, septation and polarized yeast budding and hyphal apical extension, corroborating that all these relevant biological events are Ca^{2+}-dependent processes in *E. dermatitidis* [265].

Fonsecaea pedrosoi conidial cells were able to release a metallo-type protease into the extracellular environment when grown in complex culture medium [266]. This secreted metalloprotease was active over a broad pH range, encompassing both acidic and alkaline values, it cleaved a wide range of substrates, including important human serum proteins (e.g. albumin and immunoglobulin G) and extracellular matrix components (e.g. fibronectin and laminin), and was sensitive to 1,10-phenanthroline and EGTA. The *in vitro* growth of *F. pedrosoi* conidial forms was strongly abrogated by 10 mM of each of the following chelating agents: 1,10-phenanthroline (95%), EGTA (85%) and EDTA (60%) (Fig. **7A**) [266]. Also, 1,10-phenanthroline at 1 mM concentration blocked the differentiation process from conidia into mycelia (Fig. **7B**) [266], an essential step during the *F. pedrosoi* life cycle [262].

EDTA, EGTA and 1,10-phenanthroline,in a concentration of 0.1 mM, powerfully arrested the *in vitro* growth of *C. neoformans* along of 96 h of cultivation in minimum medium (Fig. **8A**). After growth on a medium containing EDTA, the urease activity of the

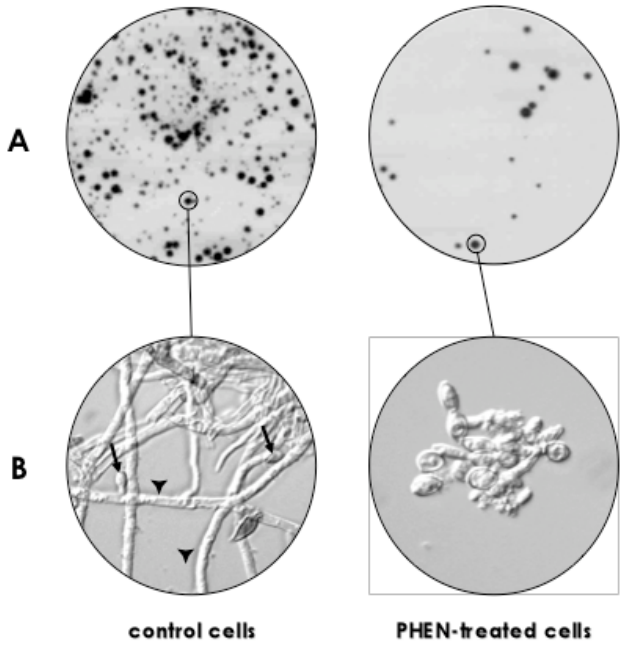

Fig. (7). Effect of PHEN on the development of *Fonsecaea pedrosoi*. Conidia (10^6) were incubated in the absence (control) or presence of 1 mM PHEN. (**A**) These mixtures were incubated for 20 h and then inoculated in fresh solid medium. Image digitalization of the plates was shown. (**B**) Optical microscopy of the *F. pedrosoi* cells after chelating treatment. Note the presence of few conidia (arrows) and many mycelia (arrowheads), showing the well-known differentiation process *in vitro*, in chelating-untreated cells. By contrast, PHEN inhibited the growth as well as the transformation of conidia into mycelia. For experimental details see Palmeira and co-workers [266].

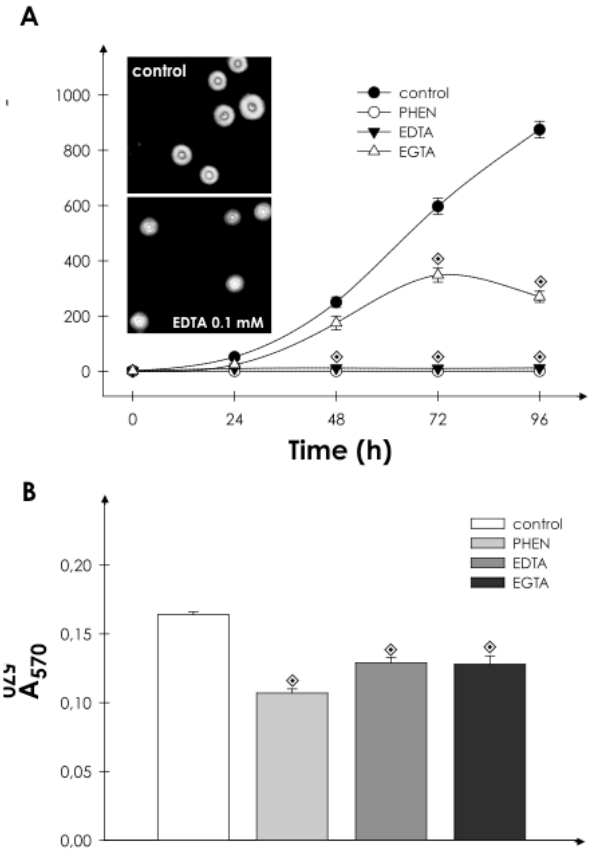

Fig. (8). Effect of chelating agents on the growth behavior, on the capsule size and on the biofilm formation by *Cryptococcus neoformans*. (**A**) Yeasts (10^5) were incubated up to 96 h in the absence (control) or presence of 0.1 mM of the following chelating agents: PHEN, EDTA or EGTA. The inset in (**A**) shows the images of the most-representative capsule size-related events observed in untreated cells (very large capsules) and EDTA-treated cells. (**B**) In order to measure the biofilm formation, yeasts were plated to adhere to the polystyrene substrate in the absence (control) or presence of different chelating agents at 0.1 mM and then the systems were incubated for 48 h. Biofilm formation was quantified by crystal violet staining. *P* values of 0.05 or less were considered statistically significant in relation to the control (◈, Student's t-test).

isolates of *C. neoformans* var. *gattii* was suppressed, while that of isolates of *C. neoformans* var. *neoformans* variety was not. This phenomenon is another biological distinction between the two varieties of *C. neoformans* [267]. EDTA, monovalent ions or excessive concentrations of Ca^{2+}ions were capable of reducing the viscosity of the major virulence factor expressed by this fungus, the capsule, supporting the role of divalent metals on polysaccharide capsular aggregation in *C. neoformans* [268].In this sense, EDTA-treated *C. neoformans* yeast cells had their capsule size considerably reduced in comparison with the untreated yeasts (Fig. **8A, inset**). Tesfa-Selase and Hay [269] purified and characterized a manganese-containing superoxide dismutase in *C. neoformans*, which was inhibited by SDS, sodium azide, 1,10-phenanthroline and EDTA, in descending order of activity. Chelating agents also perturb the adhesion properties of *C. neoformans* yeast cells, diminishing its capability to form biofilm in a plastic support (Fig. **8B**).

Deprivation of calcium ions by the addition of EGTA to culture media inhibited the growth of *H. capsulatum* mycelial cells in a dose-dependent fashion, but had no effect on yeast growth *in vitro* in a calcium-poor medium. The simultaneous addition of equimolar amounts of EGTA and $CaCl_2$ allowed normal growth of the mold form. Curiously, only the yeast form was able to release a calcium-binding protein [270]. Extracellular collagenolytic metalloprotease was reported in *H. capsulatum*, which presents an inhibition profile by EDTA [271]. In a minimal basal medium with glucose at pH 4.0 and 25°C, a lowering of the magnesium and zinc ion concentrations or an increase in calcium ion concentration of the medium favored the yeast-into-mycelium transition in *Sporothrix schenckii*. Corroborating these results, the addition of EDTA (5 mM) or EGTA (20 mM) to the culture medium delayed germ tube formation in *S. schenckii* [272].

1,10-Phenantroline at 0.5 μM induced a delay in the initiation of growth of the yeast and almost total inhibition of the mycelium of *Paracoccidioides brasiliensis* [273]. The dimorphic transition from mycelium into yeast is triggered by a temperature shift from 25°C to 37°C and is critical for *P. brasiliensis* to establish the disease [274]. Intracellular Ca^{2+}ion levels increased in hyphae immediately after temperature-induced dimorphism. The increase of cytoplasmic free Ca^{2+}ions induced the activation of calcineurin, which is a Ca^{2+}/calmodulin-dependent, serine/threonine-specific phosphatase essential for adaptation to environmental stresses, growth, morphogenesis and pathogenesis in many fungal species [275]. The chelation of Ca^{2+}ion with extracellular (EGTA ranging from 0.1to 10 mM) or intracellular (BAPTA at 5 μM) calcium chelators inhibited temperature-induced dimorphism, whereas the addition of extracellular Ca^{2+} accelerated the dimorphism process [276, 277].

Silva and co-workers [278] examined the effect of chelating agents on the *S. apiospermum* growth behavior under the condition of varying cell density. The treatment of 10^2–10^6 conidia with 10 μM EGTA did not significantly alter the growth of *S. apiospermum* compared with that of non-treated cells. Similarly, no modification

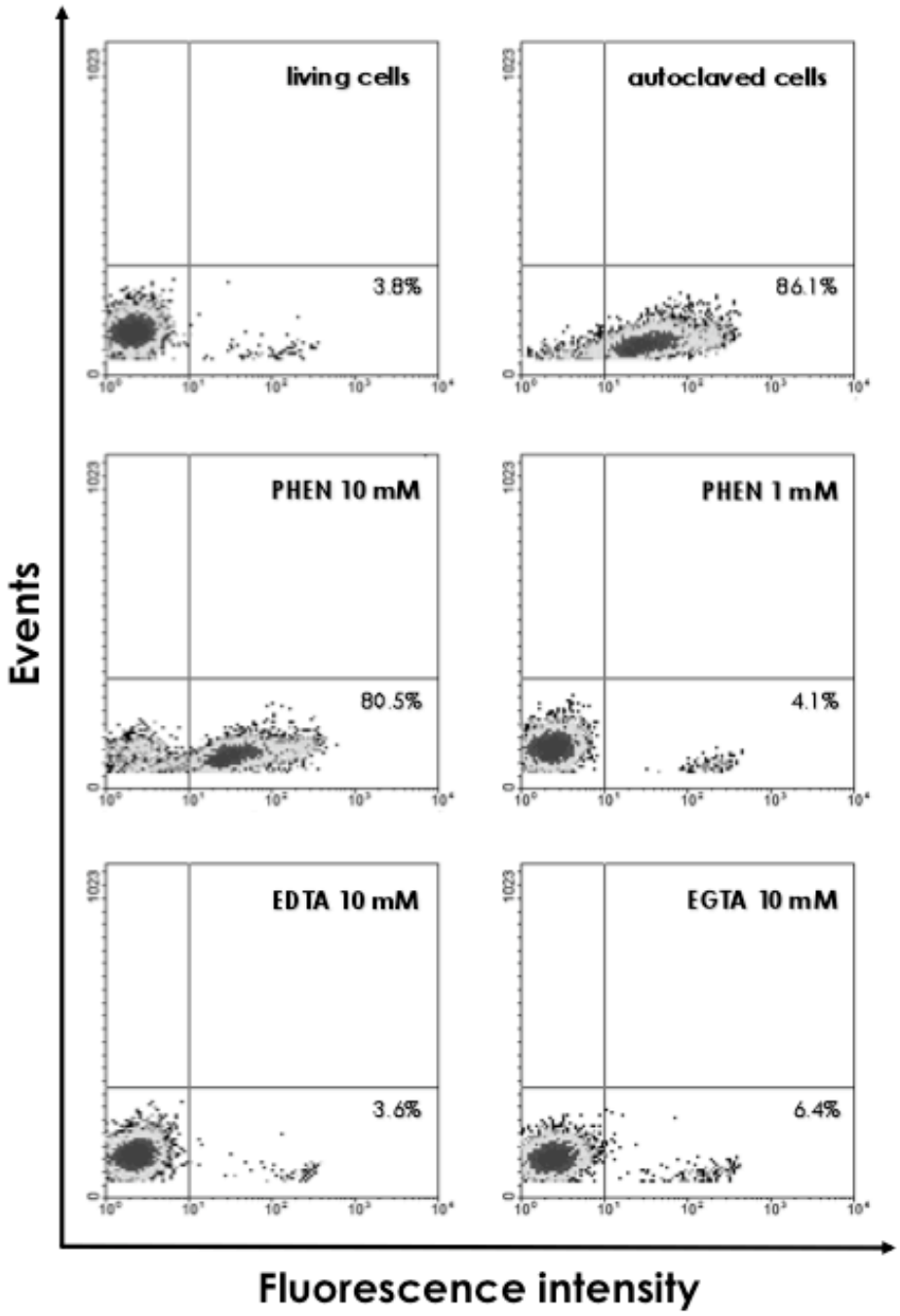

Fig. (9). Action of chelating agents on the viability of *Scedosporium apiospermum*. Conidia (10^6) were incubated for 24 h in the absence (living cells) or presence of PHEN, EDTA or EGTA. Fungal viability was measured by propidium iodide incorporation through flow cytometry analysis. Autoclaved conidia were used as control of fungal death. For experimental details see Silva and co-workers [278].

in the cell number was observed when 10^3–10^6 conidia were pre-incubated with EDTA and 1,10-phenanthroline. Conversely, growth was completely inhibited when 10^2 conidia were incubated with 10 μM 1,10-phenanthroline, while EDTA inhibited the growth by approximately 65%, showing a fungicidal action which was dependent on the cell number. The membrane permeability of *S. apiospermum* conidia (10^6 cells) was evaluated after incubation for 20 h with these chelating agents. The results showed that only 1,10-phenanthroline at 10 mM was able to induce a higher incorporation of propidium iodide (around 80%) by conidial cells, as judged by flow cytometry analyses (Fig. **9**). In addition, treatment of *S. apiospermum* with chelating agents, especially 1,10-phenanthroline and EDTA, reduced significantly the amount of ergosterol in the fungal membrane (Fig. **10A,B**), which must be related to changes in the membrane fluidity. These distinct chelating agents were also able to arrest the transformation of *S. apiospermum* conidia (10^6 cells) into hyphae in different ways; 1,10-phenanthroline completely blocked this process at 10 μM, while EDTA and EGTA only partially inhibited the differentiation at up to 10 μM [278]. At this cellular density the chelating agents did not inhibit the conidia viability, showing a direct effect on blockage of differentiation. During the conidia into mycelia differentiation process, several proteins ranging from 15 to 100 kDa were released into the culture medium by *S. apiospermum*. Interestingly, the pre-treatment of

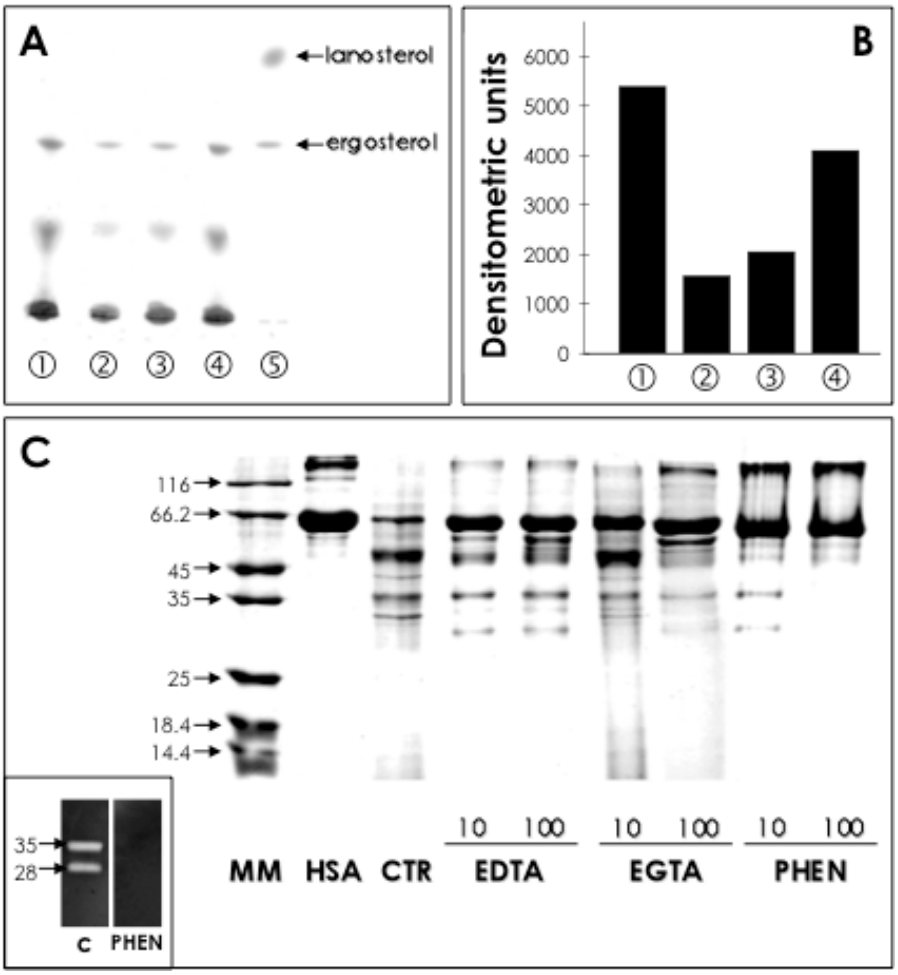

Fig. (10). Effect of chelating agents on the expression of sterol and on the hydrolytic activity of metalloproteases secreted by *Scedosporium apiospermum*. (**A**) Conidia (10^6) were incubated for 24 h in the absence (①) or presence of 1 mM of the following compounds: PHEN (②), EDTA (③)or EGTA (④). The sterol content was chemically extracted and partitioned according to Folch and co-workers [290]. The lower phase, containing neutral lipids, was recovered for analysis by high-performance thin-layer chromatography (HPTLC). A mixture of two sterol standards (ergosterol and lanosterol) were applied into the same plate (⑤). (**B**) Densitometrical analyses of the ergosterol-corresponding bands, expressed in arbitrary units. (**C**) Effect of chelating agents on the cleavage of soluble human serum albumin (HSA) by *S. apiospermum* mycelial-derived extracellular metalloproteases. Concentrated supernatant was incubated in the absence (CTR) or presence of different concentrations of EDTA, EGTA and PHEN. A control (line HSA) in which the HSA was supplemented only with buffer (pH 5.5) was used. The numbers on the left indicate apparent molecular mass of standard proteins (MM). The inset in (**C**) shows the secreted proteases by *S. apiospermum* mycelia evidenced by SDS-PAGE containing HSA as the co-polymerized substrate. For experimental details see Silva and co-workers [279, 280].

conidia with 10 μM 1,10-phenanthroline totally inhibited the secretion of polypeptides, while EDTA partially blocked this process. Conversely, EGTA-treated conidia were able to release polypeptides into extracellular surroundings similarly to the untreated cells [278]. Mycelial cells of *S. apiospermum* were able to release metallo-type proteases (28 and 35 kDa) restrained by 1,10-phenanthroline during their *in vitro* growth (Fig. **10C**, inset) [279, 280]. Additionally, cell-associated metalloproteases were detected in both conidial and mycelial extracts [281].Quantitative protease assay, using soluble albumin, showed a higher metalloprotease production in mycelial cells in comparison with conidia. In this sense, conidia synthesized a single protease of 28 kDa, while mycelia yielded 6 distinct metalloproteases in the range 28-90 kDa [281]. The regulated expression of zinc-dependent metalloproteases in the different morphological stages of *S. apiospermum* represents a potential target for the action of 1,10-phenanthroline in order to block the differentiation process as well as the fungal nutrition capability. Corroborating these findings, the 28- and 35-kDa metalloproteases were able to cleave different soluble proteinaceous substrates such as extracellular matrix components (laminin and fibronectin), sialylated proteins (mucin and fetuin) and serum proteins (albumin, hemoglobin and immunoglobulin G) (Fig. **10C**) [280], which may help the fungus to escape from natural human barriers and defenses.

5. CONCLUSIONS

Interfering in ion-dependent processes in fungi and protozoa may be an interesting approach to defeat these microorganisms. Iron is crucial for all living organisms since it is involved in a wide variety of important metabolic processes and pathogenesis. Therefore, a successful pathogen, among several characteristics, must also be able to effectively uptake iron to achieve success in the highly iron-restricted environment of the host's tissues and body fluids. Additionally, the metalloenzymes, which usually requires zinc, magnesium or calcium for activity, are widely distributed and play crucial roles in a diverse range of biochemical pathways and processes, thus a number of potential antimicrobial enzyme targets have been described. It should be taken on account that metal ions are crucial in every cellular system, including the host. Therefore,

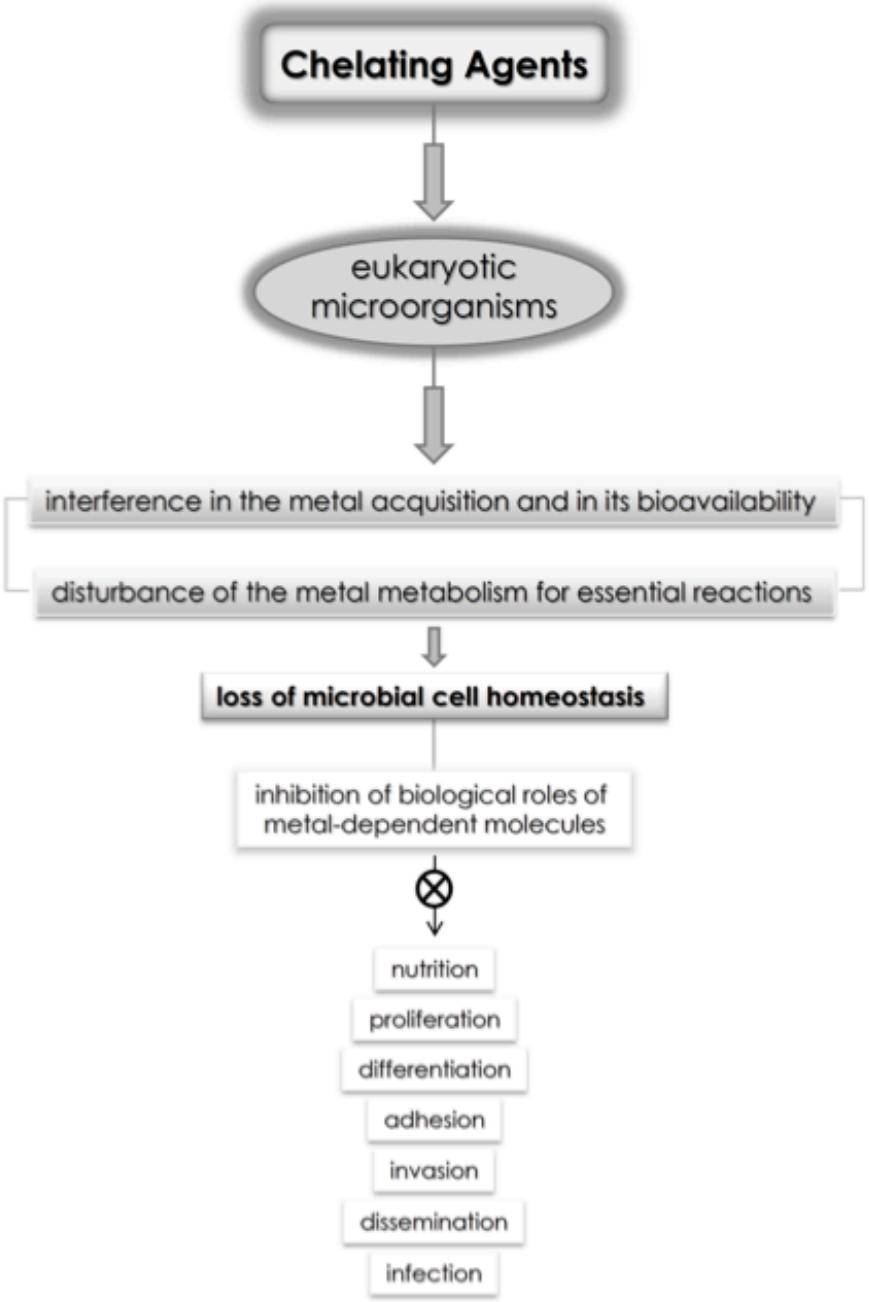

Fig. (11). Anti-microbial mechanisms of chelating agents. These compounds disturb the crucial metal metabolism of the microorganism by interfering with metal acquisition and its bioavailability for crucial reactions. In general, the chelating agents are able to inhibit the functions of metal-dependent molecules (e.g., metalloproteins), disturbing the microbial cell homeostasis and culminating in the blockage of primordial biological events such as nutrition, proliferation, differentiation, adhesion, invasion, dissemination and infection.

an intervention aiming to treat an infection through ion sequestration must deal with the delicate balance between positive and negative effects both in the pathogen and on the host. Future work should focus on the development of non-toxic ion chelators with prolonged biological half-lives and selectivity for microorganism uptake. Such compounds may provide a new class of anti-protozoan or anti-fungal drugs specifically interfering with the critical ions metabolism of the pathogen. In this sense, several studies have shown that metal-chelating agents are able to inhibit essential biological process of several eukaryotic microbial cells such as nutrition, growth, proliferation, differentiation as well as relevant pathological events like adhesion to host structures, adhesion to abiotic surfaces, evasion of host immune response and the up-regulation of virulence factors. So, the use of chelating agents to control the *in vitro* and *in vivo* microbial development is a plausible chemotherapeutic alternative. Moreover, ion chelators may be of interest for combination therapy with existing anti-protozoan or anti-fungal compounds. Therapeutic strategies involving combinations of selective chelators and other classical antimicrobial drugs may possibly prove beneficial in the treatment of infections. The use of a combination of chelating agents has been shown its usefulness and may reduce the incidence of chelating agent toxicity and also delay the emergence of drug resistance. Collectively, all these data support clinical investigation of

adjunctive chelation therapy to improve the poor outcomes with current therapy used to combat both fungal and protozoan infections.

In this sense, several studies have shown that metal-chelating agents are able to inhibit essential biological process of several eukaryotic microbial cells such as nutrition, growth, proliferation, differentiation as well as relevant pathological events like adhesion to host structures, adhesion to abiotic surfaces, evasion of host immune response and the up-regulation of virulence factors (Fig. (**11**)).

CONFLICT OF INTEREST

None declared.

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ABREVIATIONS

1-C-Grx	=	monothiol glutaredoxins
BAL	=	British-Anti-Lewisite (dimercaprol)
BAPTA	=	bis-(o-aminophenoxy)-ethane-N,N,N′,N′ tetraacetic acid
BAT-TM/BAT-TE	=	diethyl and dimethyl forms of ethane-1,2-bis (N-1-amino-3-ethylbutyl-3-thio)
BCS	=	2,9- dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulphonic acid disodium salt
BPDS	=	bathophenanthroline sulphonate
BZ	=	benznidazole
CP94	=	1,2-diethyl-hydroxypyridin-4-one
DA	=	diminazene aceturate
DFO	=	deferrioxamine B
DPA	=	D-penicillamine
EDDA	=	ethylenediamine-di(o-hydroxyphenylacetic acid)
EDTA	=	ethylenediaminetetraacetic acid
EGTA	=	ethyleneglycoltetraacetic acid
FDA	=	Food and Drug Administration
FR160	=	N4-nonyl,N1,N8-bis(2,3-dihydroxybenzoyl) spermidine hydrobromide
HEEDTA	=	hydroxy-2-ethylenediaminetriacetic acid
HMWM	=	high-molecular-weight mannoproteins
IFN-γ	=	interferon-γ
IL	=	interleukin
L1	=	1,2-dimethyl-hydroxypyridin-4-one
LAmB	=	liposomal amphotericin B
MFC	=	minimum fungicidal concentration
MIC	=	minimum inhibitory concentration
NO	=	nitric oxide
PCV	=	packed cell volume
PIST	=	post-infection survival time
Quin-2	=	2-{[2-bis(carboxymethyl)-amino-5-methylphenoxy]methyl}-6-nethoxy-8-bis(carboxymethyl)aminoquinoline
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAT	=	N′,N′,N′-tris(2-methyl-2-mercaptopropyl) 1,4,7-triazacyclononane

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