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Full Length Research Paper

In vitro parasite count and EC_{50} in trypanosome cultures incubated with some selected iron chelators

Sulaiman Faoziyat A.[1]*, Akanji M.A[1], Green, B. O[2], Badjatia, N[3] and Gunzl A[3]

[1]Biochemistry Department, University of Ilorin, PMB 1515 Ilorin, Nigeria.
[2]College of Naturopathic Medicine, University of Bridgeport, Connecticut, U.S.A.
[3]Genetics and Developmental Biology, University of Connecticut, Farmington USA.

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***Trypanosoma brucei* (Lister 427 strain) grown in *in vitro* cultures were incubated with some selected iron chelators. Parasite counts were done at 24hrs interval for 72hours to determine their effectiveness on parasites' reduction. The EC_{50} for each drug in the management of trypanosomiasis was also determined. While deferoxamine, deferiprone and adriamycin had the most significant effect ($P < 0.05$) on parasite reduction, Inositol heaxphosphate and Magnesium trisilicate had the least. Magnesium trisilicate was suggested to be a potent growth factor for the parasites rather than as inhibitor since no EC_{50} could be calculated. The EC_{50} for the drugs were calculated to be 8.02µM, 13.71µM, 27.30µM, 420.98µm, and 1404.02µM, for Deferoxamine, Deferiprone, Adriamycin, ibuprofen and Inositol hexaphosphate respectively. The order of efficacy of the drugs in the management of trypanosomiasis is thus: Deferoxamine > Deferiprone > Adriamycin > Ibuprofen > Inositol Hexaphosphate > Mg. Trisilicate**

Keywords: Chelators, Deferoxamine, Deferiprone, Adriamycin, Ibuprofen, Magnesium trisilicate, inositol hexaphosphate Trypanosome culture, EC_{50}**.**

INTRODUCTION

Trypanosoma brucei is the causative agent of African sleeping sickness (Trypanosomiasis) in humans and livestocks where they exert severe morbidity and mortality (Eisler *et al.,* 2003). The parasites live extracellularly in blood and tissue fluids of mammals and are transmitted by the bite of infected tsetse fly (*Glossina* spp.) (WHO, 2011). Human African Trypanosomiasis (HAT) is a widespread African tropical disease that can be fatal if not treated.

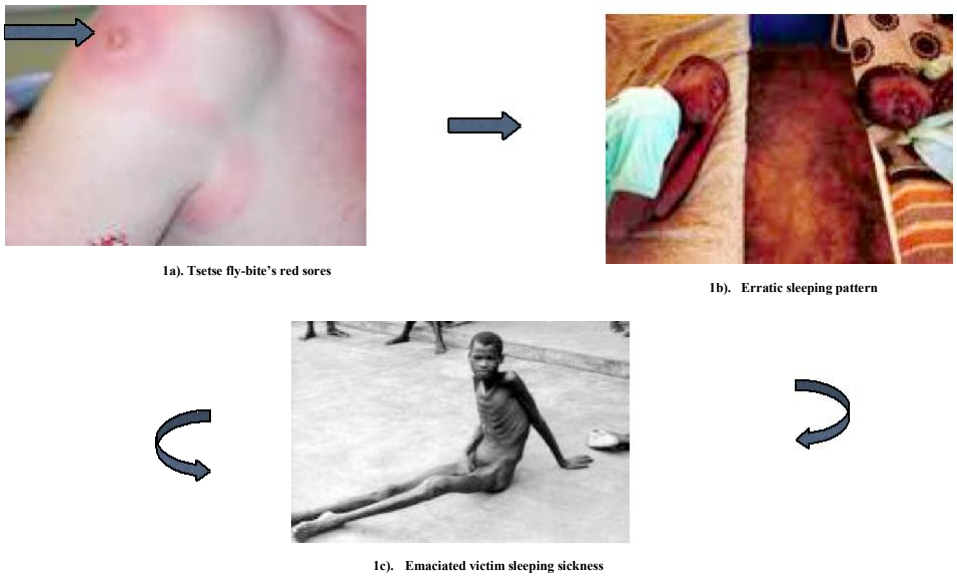
The tsetse fly bite erupts into a red sore (Figure 1a), the parasites then multiplies in the blood and lymph glands, and within a few weeks, the person can experience fever, swollen lymph glands and aching muscles among others. In advanced stages, the disease affects the central nervous system, the parasites cross the blood-brain barrier and provokes major, often irreversible, neurological disorders causing changes in personality, alteration in sleeping pattern (Figure 1b), alteration in the biological clock (the circadian rhythm), confusion, slurred speech, seizures, and difficulty in walking, talking and emaciation (Figure 1c) (CDC, 2010.)

Ribonucleotide reductase (RNR) is an iron-containing enzyme that is essential for DNA synthesis, it require two iron co-factors for its optimum activity, which are the target of the iron chelators used in this study. The relevance of these studies is therefore to identify potential trypanosomal drugs for the management of the disease.

MATERIALS

Parasites: Trypanosoma brucei bloodstream (Lister 427 strain) cell culture was used for the *in vitro* targeted integration of linear DNAs in the RNAi experiments and generation of stable cell lines were obtained and used in Prof. Gunzl's lab. at the Department of Genetics and Developmental Biology, University of Connecticut, Farmington, USA. All experiments were conducted within a sterile environment in the cabinet or hood of Labconco Purifier class II Biosafety Cabinet, to prevent contamination.

*Corresponding author E-mail: faoziyat20022002@yahoo.com



1a). Tsetse fly-bite's red sores

1b). Erratic sleeping pattern

1c). Emaciated victim sleeping sickness

Figure 1. Tsetse fly-bite's red sores, erratic sleeping pattern and emaciated victim of sleeping sickness.
Sources: David *et al.* (2002); CDC (2010a, 2010b).

Drugs of interest: Deferiprone, Inositol hexaphosphate, Ibuprofen, Deferoxamine Mesylate salt, Magnesium trisilicate and Adriamycin (Doxorubicin Hydrochloride) were products of Sigma-Aldrich, Missouri, USA.

METHODS

In vitro anti-trypanosomal studies: 6 of 24 culture well plates were used to incubate each drug with the parasites for 72 hours to determine not only their anti-trypanosomal effect but also their EC_{50}.

Determination of Half maximal effective concentration (EC_{50}): The term half maximal effective concentration (EC_{50}) refers to the concentration of a drug, antibody or toxicant, which induces a response halfway between the baseline and maximum after some specified exposure time. It is commonly used as a measure of drug's potency. Logistic regression method by linear interpolation was used as described by Hills *et al.,* (1986) and modified by Huber and Koella, (1993).

$$\log (EC_{50}) = \log (x1) + \frac{y_1 - y_0/2}{y_I - y_2} (\log (X_2) - \log (X_1))$$

RESULTS AND DISCUSSION

The parasite counts were monitored over 72 hours in trypanosome cultures incubated with deferoxamine (figure 2), inositol hexaphosphate (IP_6) (figure 3) and magnesium trisilicate (figure 4) as well as those incubated with ibuprofen (figure 5), deferiprone (figure 6) and adriamycin (figure 7).

Parasite count

Deferoxamine growth curve at 20 µM showed the least parasite count. No parasite was visibly mobile in the IP_6 culture at 3000 µM by 72 hours. For magnesium trilsilicate, result showed the 500 µM group had higher parasite count than 300 µM, 400 µM and the control groups. A significant decrease ($P < 0.05$) in the parasite count was observed at 40 µM at 72 hours post incubation with Deferiprone (figure 6) and Adriamycin (figure 7). For Ibuprofen, major significant decline in parasite count was observed at 500 µM (Figure 5). The significant increase ($P < 0.05$) in parasite count in magnesium trisilicate-incubated groups (Figure 4) indicated that it might be a potent growth factor, aiding the growth of the parasites rather than inhibiting it. However, significant reduction in parasite count observed in groups incubated with deferoxamine, deferiprone, adriamycin, ibuprofen, and inositol hexaphosphate is probably as a result of their potentials to reduce, chelate or mop up iron, thereby reducing parasites' access to the vital cofactors they required for survival.

EC_{50} VALUES

EC_{50} values for Deferoxamine and IP_6

Deferoxamine, the control drug was incubated with the parasites at various concentrations, not only to monitor the effect of the drug on the parasites' growth but also to calculate the EC_{50} (Huber and Koella, 1993). In a series of repeated experiments 1 to 20 µM of deferoxamine (figure 8) were incubated with the parasites and the first

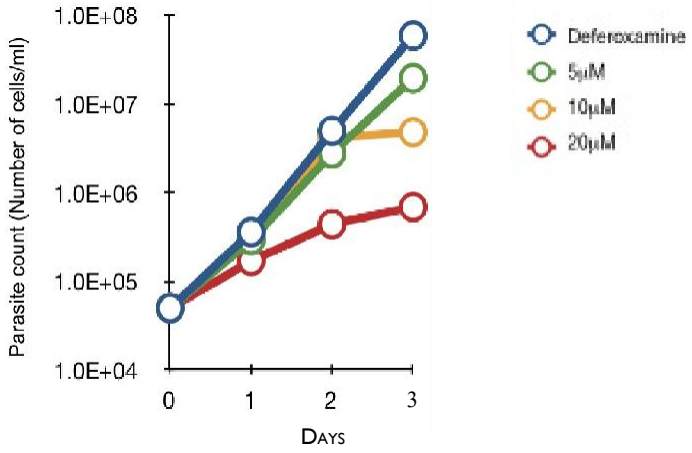


Figure 2. Effect of deferoxamine on parasite count

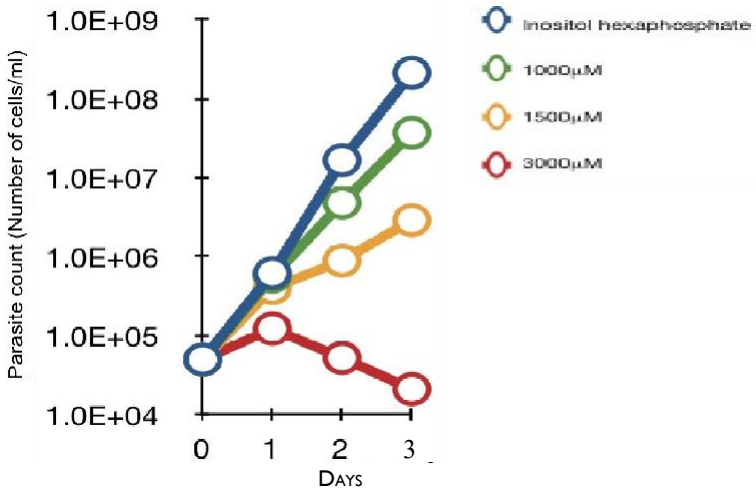


Figure 3. Effect of inositol hexaphosphate on parasite count

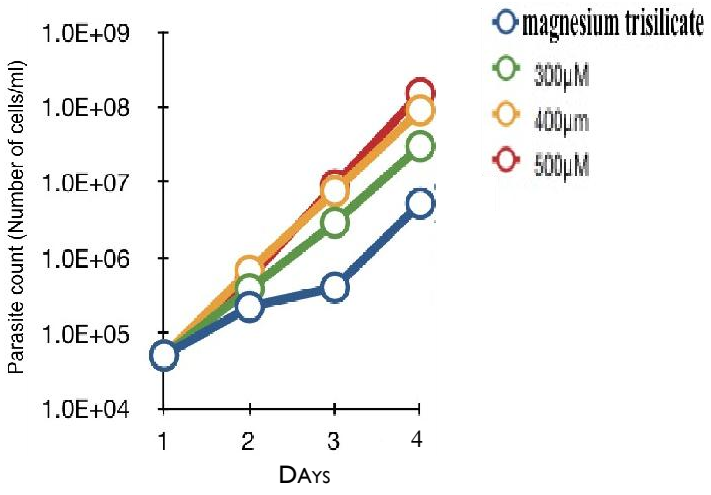


Figure 4. Effect of magnesium trisilicate on parasite count

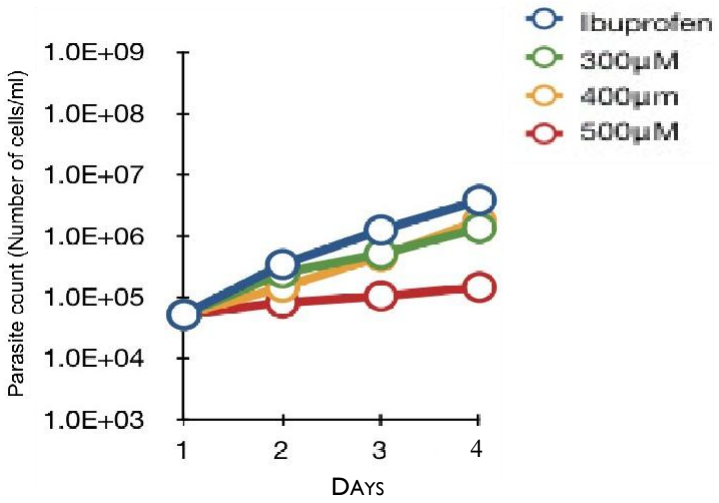


Figure 5. Effect of ibuprofen on parasite count

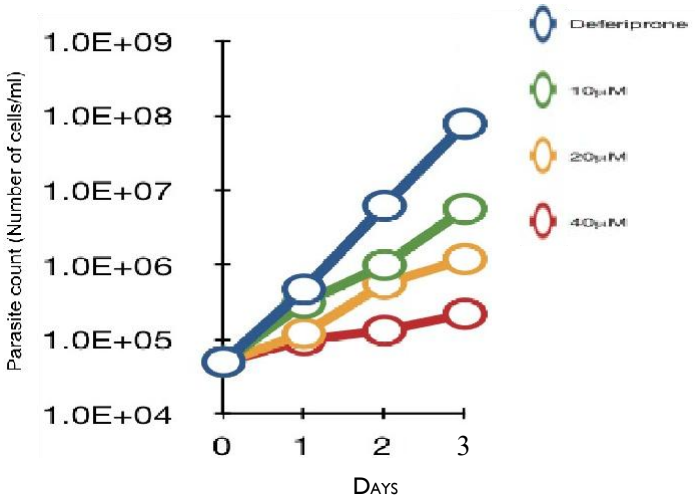


Figure 6. Effect of deferiprone on parasite count

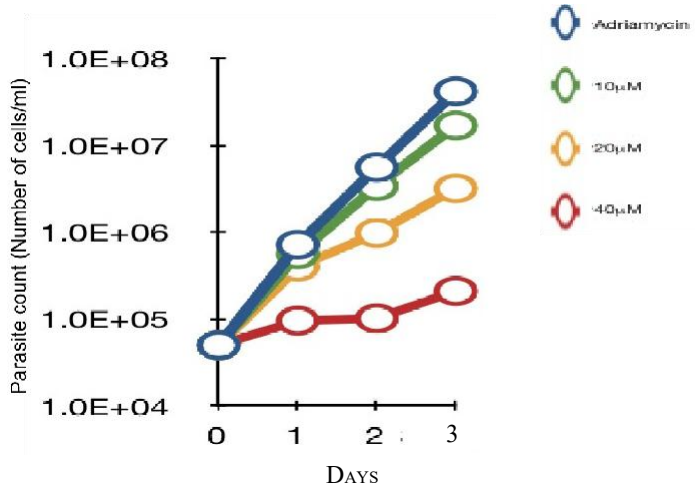


Figure 7. Effect of adriamycine on parasite count

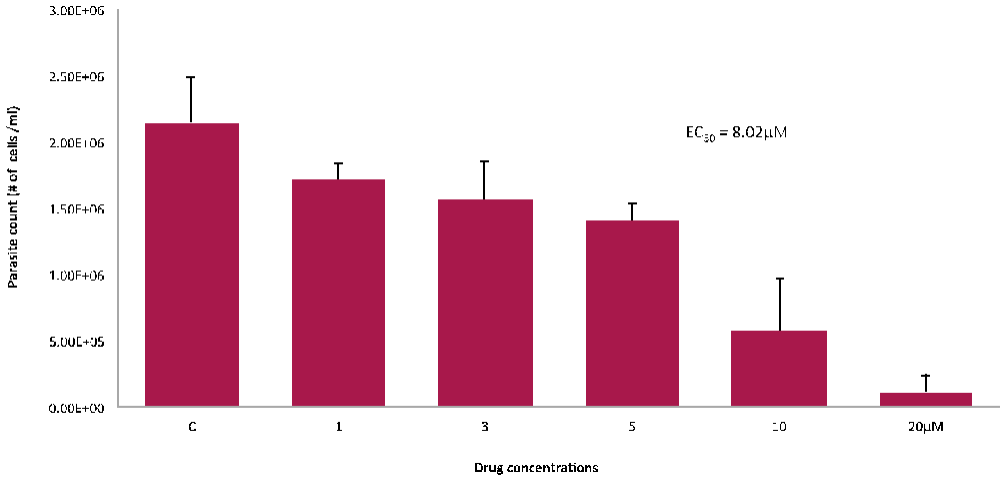


Figure 8. Parasite count in cultures incubated with deferoxamine
Each value is an average of 4 replicates

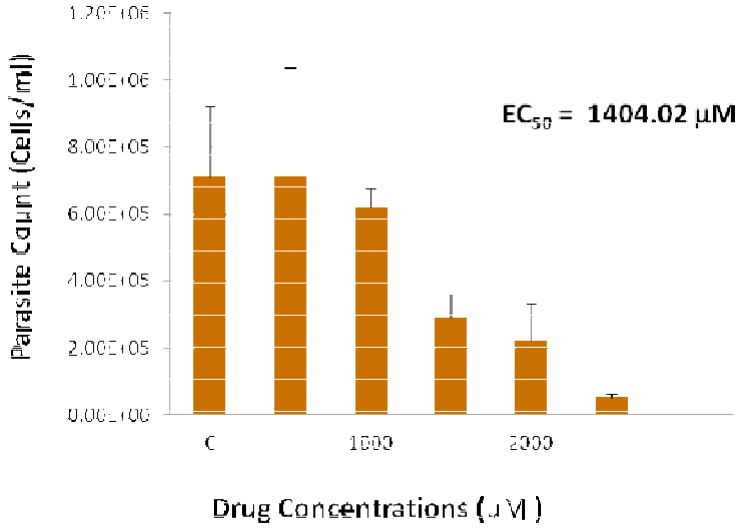


Figure 9. Parasite count in culture incubated with inositol hexaphosphate
Each value is an average of 4 replicates

significant decrease ($P > 0.05$) in the number of the parasites was observed between 5 and 10 µM, the EC_{50} was then calculated to be 8.02 µM. In earlier preliminary studies, inositol hexaphosphate (IP_6) had no significant effect on the parasite count at concentrations between 50 and 600 µM. The concentrations were increased in another experiment to between 800 and 3000 µM (figure 9). The first significant decrease was observed between 1000 and 1500 µM and the EC_{50} was calculated to be 1404.02 µM.

EC_{50} values in Ibuprofen and magnesium trisilicate

Preliminary incubations of ibuprofen with the parasite were from 50 to 500 µM. The first significant reduction ($P > 0.05$) in parasite count was noticed from 400 µM of Ibuprofen. A repetition of parasite incubation between 400 and 600 µM of Ibuprofen (figure 10) showed a significant difference between 400 and 450 µM and the EC_{50} was calculated to be 420.98 µM. Preliminary incubation of magnesium trisilicate at 50 to 300 µM had no significant effect on the parasite growth. Increase in parasite growth was however observed as the concentration was increased from 300 to 500 µM (figure 11). Using higher magnesium trisilicate concentrations (600 to 2000 µM) in another experiment significantly increased ($P < 0.05$) the parasite count and aided their growth. The EC_{50} couldn't be calculated as two parasite populations, lower and higher numbers than half the

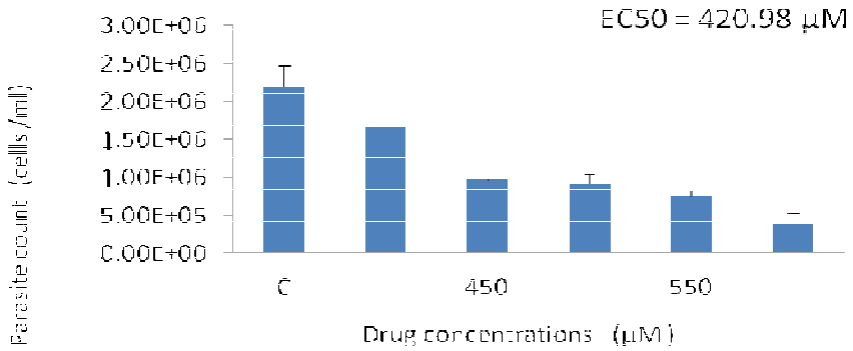


Figure 10. Parasite count in culture incubated with ibuprofen
Each value is an average of 4 replicates

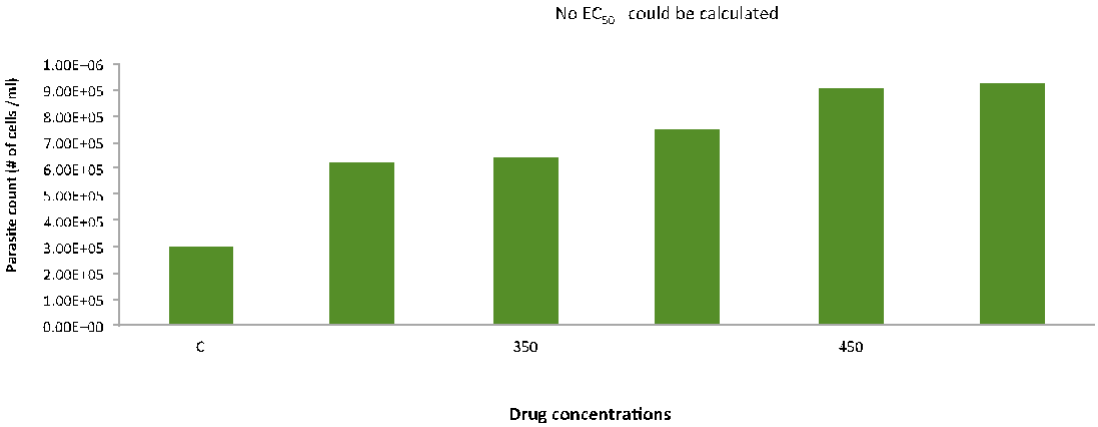


Figure 11. Parasite count in culture incubated with magnesium trisilicate
Each value is an average of 4 replicates

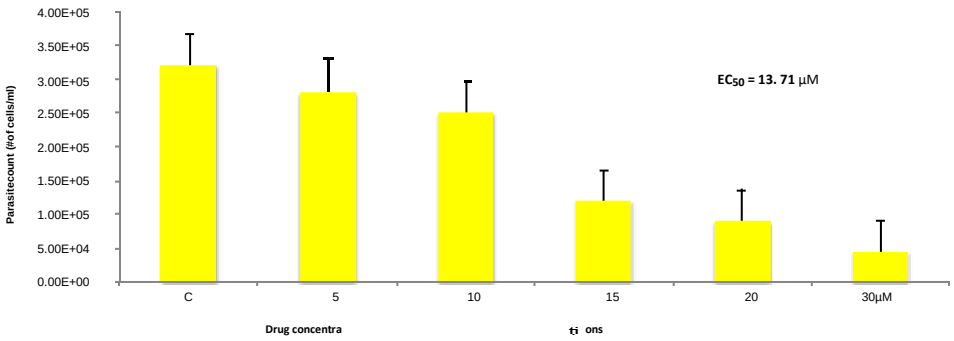


Figure 12. Parasite count in cultures incubated with deferiprone
Each value is an average of 4 replicates

population of the control are required to calculate this. All the parasite counts were higher than the control with no lower value to aid the calculation (figure 11).

EC_{50} Values for Deferiprone and Adriamycin

A significant decline and death of the parasites were observed in the first sets of trypanosomes incubation with deferiprone at concentrations between 20 and 200 µM. The EC_{50} could not be calculated, as all parasite counts were below half the population of the control. The drug solution was diluted (X10), and in another experiment with concentrations between 5 and 30 µM (figure 12), the first significant decline was observed between 10 and 15 µM and the EC_{50} was calculated to be

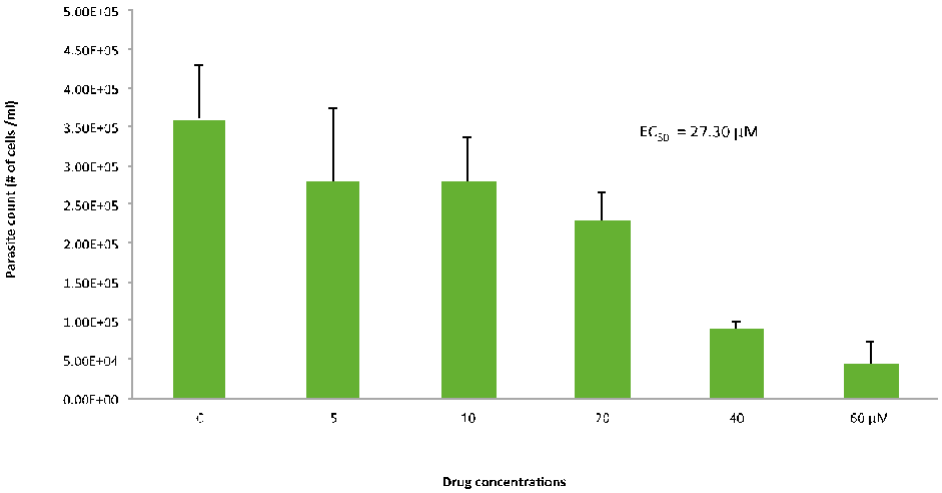


Figure 13. Parasite count in cultures incubated with adriamcyn
Each value is an average of 4 replicates

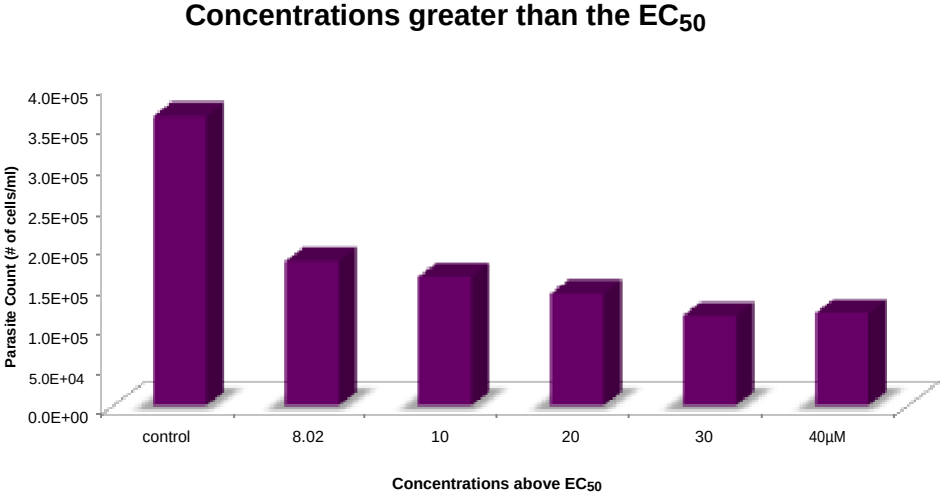


Figure 14. Parasite count in cultures incubated with deferoxamine
Concentrations above the calculated EC_{50}

13.71 µM (figure 12). 20 to 200 µM concentrations of Adriamycin (Figure 13) showed a significant decline ($P > 0.05$) in the parasites population between 20 and 40 µM. For confirmation of this result, the drug solution was diluted (X10) and re-incubated with the trypanosomes, the EC_{50} was calculated to be 27.30 µM

The lower EC_{50} obtained in Deferiprone- 13.71 µM (Figure 12) and Adriamycin – 27.30 µM (Figure 13) when compared with the standard drug, deferoxamine – 8.02 µM (Figure 8), make them better iron chelators and antitrypanosomal drugs than ibuprofen – 420.98 µM (Figure 10) and inositol hexaphosphate – 1404.02 µM (Figure 9). Merschjohann and Steverding, (2006) carried out a study on the IC_{50} values of fourteen iron chelators for bloodstream forms of *T. brucei* 427-221a and *T. congolense* STIB910, and for human myeloid leukaemia HL-60 cells, as well as calculated the IC_{50} ratios of cytotoxicity to trypanocidal activities of the chelators. Their study had only deferoxamine in common with this study, while they recorded 3.3 µM as the IC_{50} for *T. brucei,* this study computed the EC_{50} as 8.02 µM. Magnesium trisilicate (with no EC_{50} calculated) exhibited no significant tripanocidal effect, even at concentrations as high as 2000µM (Figure 11).

Another set of confirmatory experiments (figures 14 to 19) was initiated, having established the EC_{50} for each drug. This was to confirm if at higher concentrations than the EC_{50}s calculated, any of these drugs would behave like magnesium trisilicate and aid the growth of the parasites. A consistency in the parasite population

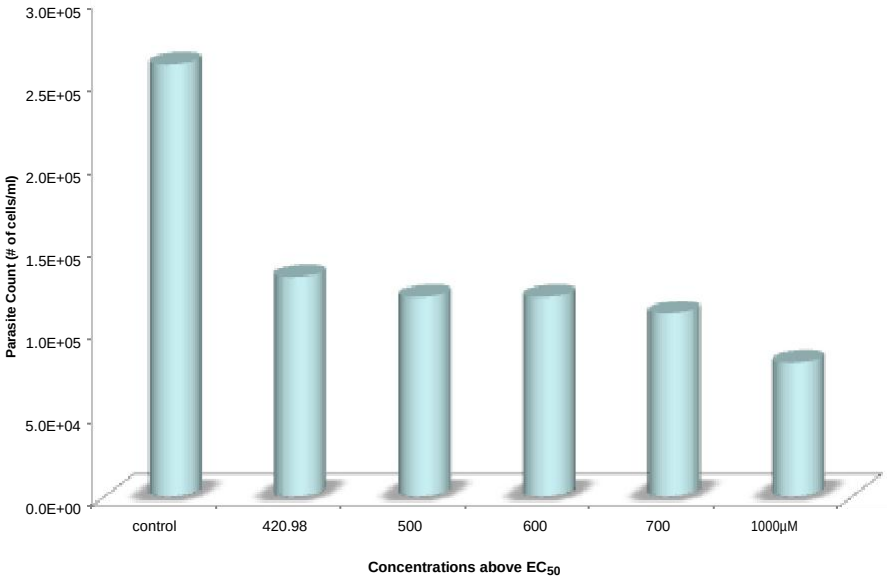


Figure 15. Parasite count in cultures incubated with ibuprofen Concentrations above the calculated EC_{50}

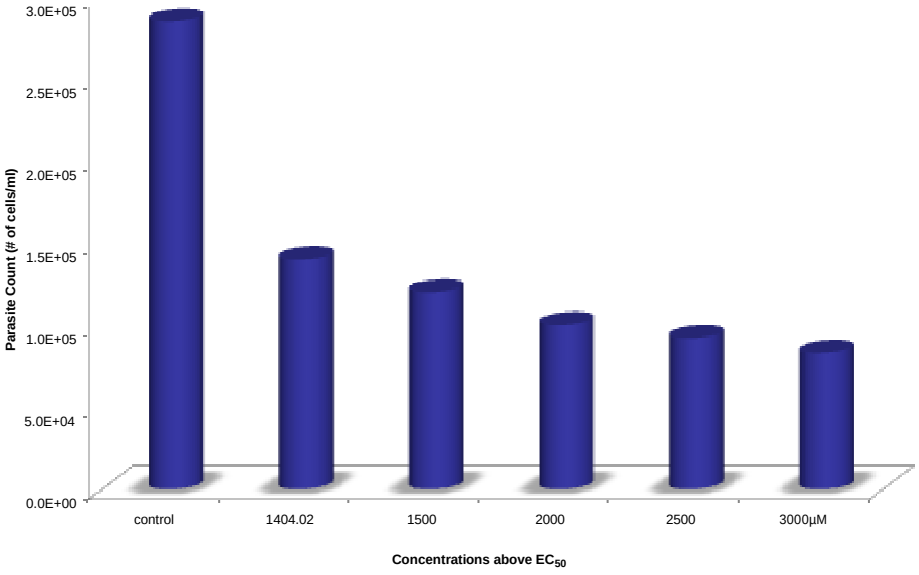


Figure 16. Parasite count in cultures incubated with inositol hexaphosphate Concentrations above the calculated EC_{50}

decline was observed in all the five experimental groups, with Deferoxamine (figure 14), Adriamycin (figure 17) and Deferiprone (figure 18) being the most effective at very low concentrations, relative to Ibuprofen (figure 15) and IP_6 (figure 16). At concentrations as high as 2000µM, magnesium trisilicate did not significantly reduce ($P < 0.05$) the parasites population but rather; significantly increase it (figure 19).

The continuous decline in parasite count ($P < 0.05$) with increase in the concentrations of the drugs above the EC_{50} (Figure 14 and 18) showed the disease can be managed in a dose-dependent manner, with adriamycin (figure 17), deferiprone (figure 18) and deferoxamine (figure 14) being better and more effective drugs at minimal concentrations. Magnesium trisilicate (with no EC_{50} calculated) exhibit no significant tripanocidal effect, even at concentrations as high as 2000µM (Figure 19). Magnesium trisilicate was therefore suggested to be a growth factor for the parasites rather than growth inhibitor.

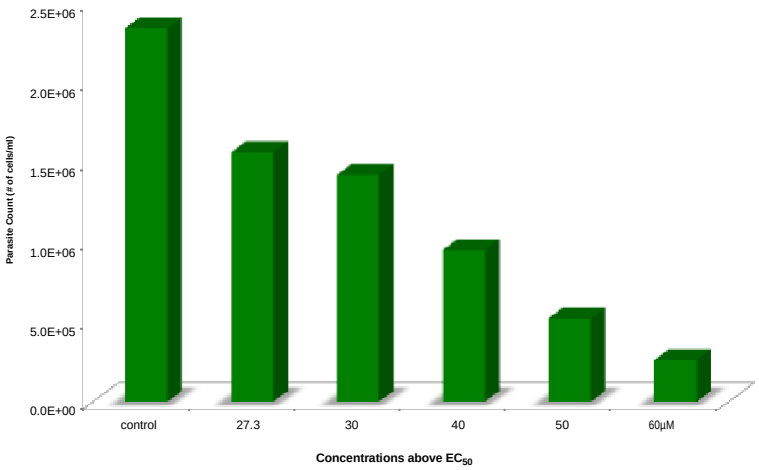


Figure 17. Parasite count in cultures incubated with adriamycin
Concentrations above the calculated EC_{50}

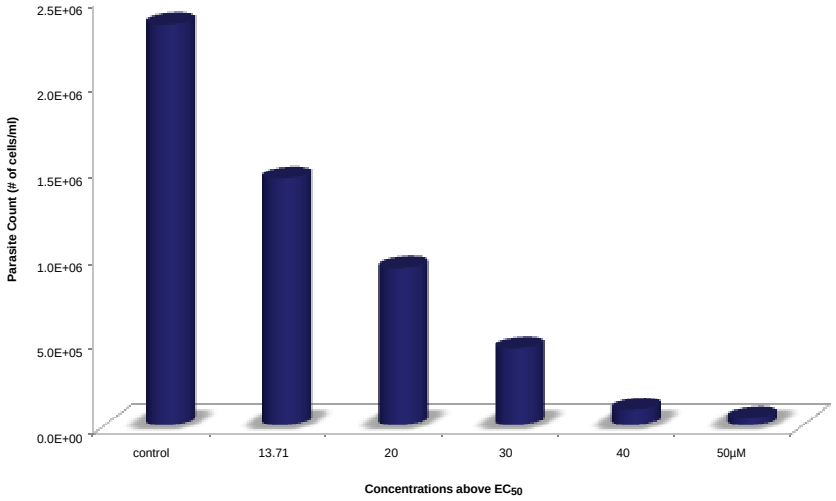


Figure 18. Parasite count in cultures incubated with deferiprone
Concentrations above the calculated EC_{50}

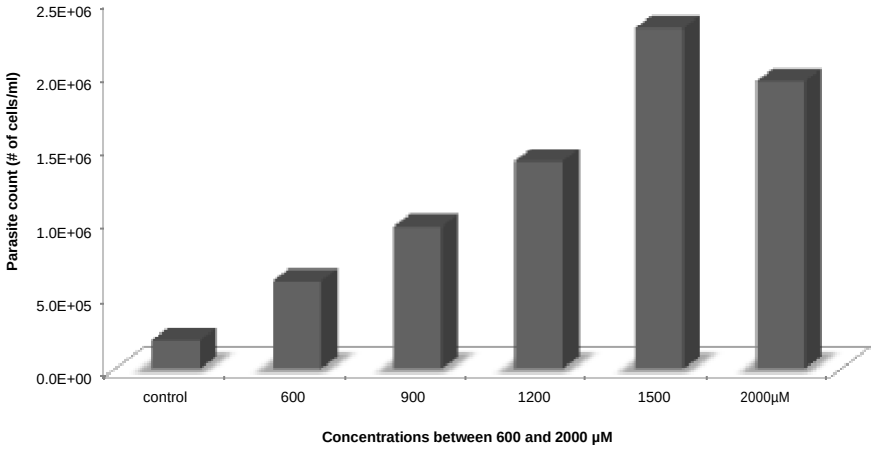


Figure 19. Parasite count in cultures incubated with magnesium trisilicate
Concentrations from 600 to 2000 µM,
EC_{50} could not be calculated despite the high concentrations used.

CONCLUSION

- The order of efficacy of the drugs in the management of trypanosomiasis is:

Deferoxamine (8.02 µM) > Deferiprone (13.71 µM) > Adriamycin (27.30 µM) > Ibuprofen (420.98 µM) > Inositol Hexaphosphate (1404.02 µM) > Mg. Trisilicate (No EC_{50}).

- Deferoxamine, Inositol hexaphosphate, Ibuprofen, Deferiprone and Adriamycin were therefore concluded to be good trypanocides while magnesium trisilicate, whose parasite count continued to increase despite significant increase in drug concentration, was regarded as a good growth factor/agent for the parasites.
- Ribonucleotide reductase was concluded to be an essential enzyme in the trypanosomes as depicted by the significant reduction in parasites' population observed and the values of EC_{50} obtained for each drug. It was also confirmed to be a very good drug target in the formulation of trypanocides.

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