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***A N I N T E R N A T I O N A L J O U R N A L***

# *In Vitro* Assessment of Antileishmanial Activity of Natamycin and Nystatin

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## ABSTRACT

*The present study was aimed to evaluate the in vitro antileishmanial activity of four different concentrations of natamycin and nystatin by using MTT 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide reduction assay. In vitro antileishmanial activity revealed that the $IC_{50}$ of natamycin (80.49 μg/ml) and nystatin (105.7 μg/ml) was less than that of sodium stibogluconate (127.9 μg/ml), and more than amphotericin B (18.91 μg/ml).*

**Key words*:*** Leishmaniasis, *Leishmania donovani*, Antileishmanial, Natamycin, Nystatin

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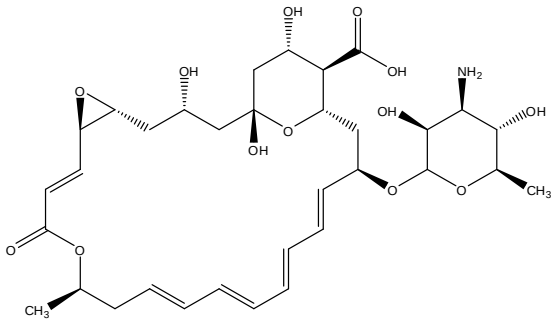
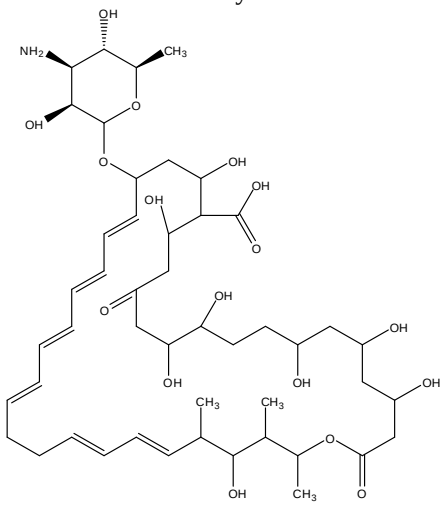
Leishmaniasis is a major public health problem in tropical and subtropical countries including India. It is caused by an intracellular obligate protozoan parasite, *Leishmania*, which spreads through the bite of infected female sand fly. It is estimated that 0.2 to 0.4 million new cases of visceral leishmaniasis (VL) and 0.7 to 1.3 million new cases of cutaneous leishmaniasis (CL) occur every year worldwide [1]. The treatment and control of leishmaniasis involves the administration of pentavalent antimonials, amphotericin B, paromomycin, pentamidine and azoles like ketoconazole [2]. Chemotherapy of leishmaniasis has been challenged by drug resistance, toxicity, variable effectiveness between species and requirement for long durations of treatment [3]. Hence, there is an urgent need to discover or develop new chemotherapeutic agents for the treatment of leishmaniasis which may reduce the unpleasant side-effects associated with conventional drugs and may control the disease safely and efficiently.

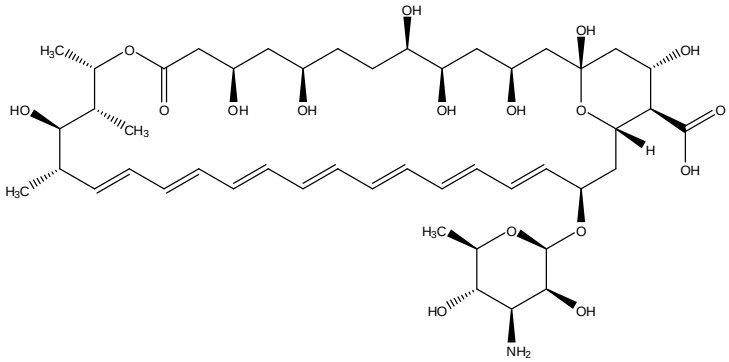
Amphotericin B is a polyene antibiotic as well as a broad spectrum antimycotic agent. It is used to cure visceral leishmaniasis cases which are non-responsive to pentavalent antimonials [4]. Despite its 97% cure rate, it has some drawbacks such as nephrotoxicity that may result in kidney failure [5,6]. Owing to the toxicity of amphotericin B and other available treatments, there is a need to develop agents with antileishmanial activity. Therefore, compounds having structural and functional similarities with amphotericin B may be screened and tested. The antileishmanial action of amphotericin B is believed to be due to its capability to bind ergosterol, a major sterol in *Leishmania* [7]. Ergosterol is found in the cell membrane of *Leishmania* and is obligatory to regulate membrane fluidity. It contributes to the organization of membrane domains. Ergosterol is not found in mammals, instead, it is replaced by cholesterol. Therefore, the compounds targeting particularly ergosterol are of great interest to inhibit *Leishmania* parasite. The antimycotic agent natamycin, a macrolide antifungal agent derived from *Streptomyces natalensis* and nystatin, a polyene antifungal drug derived from *Streptomyces noursei* are known to bind with the fungal membrane sterols and are structurally similar to amphotericin B (Figure 1). These two drugs, approved for human use, are broad spectrum antifungal agents and have never been screened against *Leishmania donovani*. The present study was aimed to evaluate the *in vitro* antileishmanial potential of natamycin and nystatin against *L. donovani*.



Natamycin



Nystatin



Amphotericin B

**Figure 1.** Chemical structures of natamycin, nystatin and amphotericin B.

The Axenic culture of *L. donovani* (LdMIPL-1) was maintained at 25°C in RPMI-1640 (Himedia, Mumbai) medium supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Himedia, Mumbai), streptomycin (150 µg/ml), penicillin G (100 µg/ml) and gentamycin (150 µg/ml) at pH 7.2 For antileishmanial activity, promastigotes of *L. donovani* were sub-cultured in Schneider's Insect Medium (Himedia, Mumbai) supplemented with 10% FBS, streptomycin (150 µg/ml), penicillin G (100 µg/ml) and gentamycin (150 µg/ml). The screening was performed in 96-well, flat bottom tissue culture plates (Corning Life Sciences, USA). A cell suspension (100 µl) containing 20-30 million cells/ml was poured in each well of the plate. Four different concentrations i.e. 25, 50, 75 and 100 µg/ml of natamycin (Sun Pharma Laboratories, Mumbai) and nystatin (Himedia, Mumbai), dissolved in dimethyl sulfoxide (DMSO) (< 0.025% v/v), were added into the culture. The plates were then incubated at 25°C for 24-48 hours. Amphotericin B (2.5 - 10.0 µg/ml) and

sodium stibogluconate (25 - 100 μg/ml) were used as positive controls and cell suspension with 0.025% DMSO was used as the negative control. The mortality of the promastigotes was assessed by measuring the cleavage of 10 mg/ml of MTT 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide [8,9]. The reduction of MTT dye results in the formation of purple formazan. Only the living cells having the enzyme oxidoreductase turn the dye into the purple compound. Thus, the intensity of the purple product formed is directly proportional to the number of living cells in the suspension. In this study, inhibition of promastigotes in the wells with different concentrations of research drugs was assessed by less formazan formed as compared to the negative control in which most of the promastigotes were alive. The absorbance was measured by using ELISA plate reader (BioTek, USA) at 595 nm. The mean percent inhibition was calculated as follows:

$$\%\ \text{of inhibition} = \frac{\text{OD control} - \text{OD treated}}{\text{OD control}} \times 100$$

Each experiment was performed in triplicate with three replicates of each concentration and the results were expressed as mean ± standard error of the mean (SEM). The $IC_{50}$ values were calculated using GraphPad Prism 5.02 software. The overall variation in a set of data was analysed by one way analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant.

The results of antileishmanial activity of the two research drugs revealed that both natamycin and nystatin possess significant antileishmanial activity in comparison to sodium stibogluconate while less activity in comparison to amphotericin B (Figure 2-3). The $IC_{50}$ values of natamycin and nystatin were 80.49 μg/ml and 105.7 μg/ml, respectively, which were less than sodium stibogluconate (127.9 μg/ml) and higher than amphotericin B (18.91 μg/ml) (Table 1).

**Table 1.** Percent inhibition of *L. donovani* promastigotes with four different concentrations of natamycin, nystatin, sodium stibogluconate and amphotericin B.

| Drug used ↓ | Percent inhibition of *L. donovani* | | | | |
|---|---|---|---|---|---|
| | 25 μg/ml | 50 μg/ml | 75 μg/ml | 100 μg/ml | $IC_{50}$ (μg/ml) |
| Natamycin | 42.27 (1.817) | 47.53 (1.34) | 49.59 (0.893) | 51.07 (1.123) | 80.49 |
| Nystatin | 38.72 (1.618) | 42.47 (2.313) | 46.23 (1.727) | 50.76 (0.956) | 105.7 |
| Sodium Stibogluconate | 30.84 (1.257) | 36.94 (1.663) | 42.87 (0.690) | 47.62 (0.506) | 127.9 |
| Amphotericin B | 41.28 (1.084) (at 2.5 μg/ml) | 41.03 (2.039) (5.0 μg/ml) | 46.18 (1.935) (7.5 μg/ml) | 47.52 (1.475) (10.0 μg/ml) | 18.91 |

*Standard error of the mean (SEM) is shown in brackets.

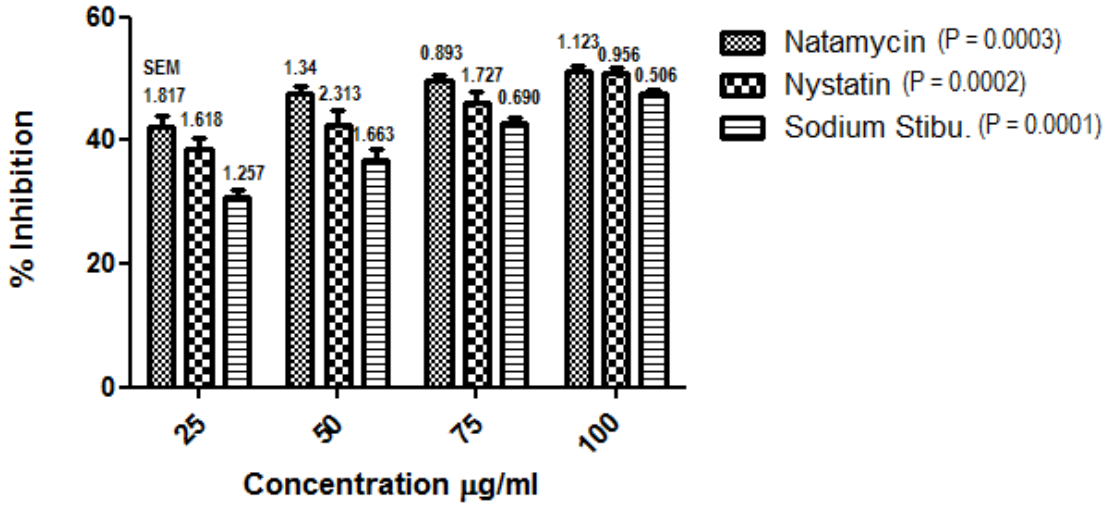



**Figure 2.** Percent inhibition of *L. donovani* promastigotes by natamycin, nystatin and sodium stibogluconate after 24 hours of incubation at 25°C and standard error of the mean. Differences were considered statistically significant (P<0.05) when comparing the parasites treated with four different concentrations i.e., 25, 50, 75 and 100 µg/ml of natamycin, nystatin and sodium stibogluconate.

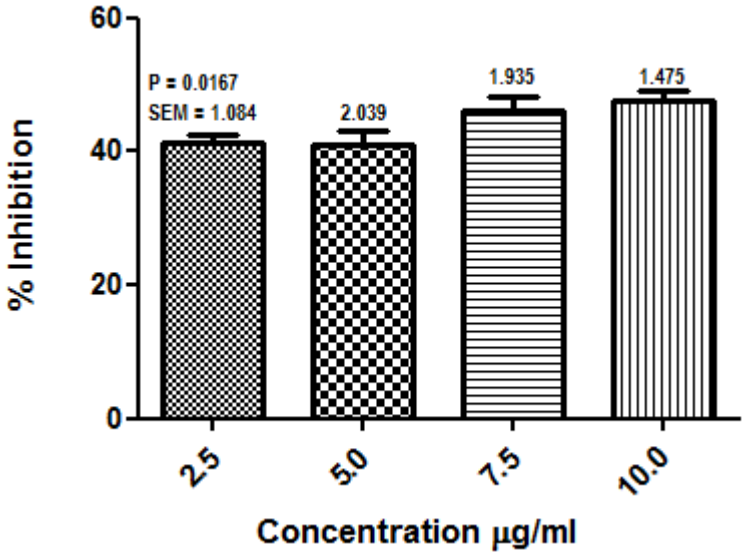



**Figure 3.** Percent inhibition of *L. donovani* promastigotes by amphotericin B after 24 hours of incubation at 25°C. Differences were considered statistically significant (P<0.05) when comparing the parasites treated with four different concentrations i.e. 2.5, 5.0, 7.5 and 10.0 µg/ml of amphotericin B.

Research is going on worldwide to find new and better antileishmanial agents of natural/synthetic origin. A recent study suggested that the use of antiretroviral drugs individually and in combination with miltefosine was effective against *Leishmania infantum*. Out of the six tested drugs, efavirenz and delavirdine mesylate individually showed $IC_{50}$ values of 26.1 µM and 136.2 µM respectively. On combining efivirenz with miltefosine the $IC_{50}$ value decreased to 11. 8 µM [10]. This is a fine idea put in the pipeline which may increase the effectiveness against the parasite while decreasing the common side effects of the previously used drug. In another study, the antileishmanial effect of twelve indolyl-coumarin hybrids was assessed and it was observed that a total of three compounds possessed significant antileishmanial activity with $IC_{50}$ values in the range of 95-99µg/ml [11]. Despite the extensive research for better antileishmanial agents, the efficacy of natamycin and nystatin,

individually or in combination with other drugs, has not been studied against *L. donovani*. Several factors such as mechanism of action against fungi i.e. binding with ergosterol and causing pores in the cell membrane, easy availability, very less toxicity as compared to amphotericin B and approval for human use prompted us to select these two drugs. In the present study, we have evaluated the effectiveness of natamycin and nystatin *in vitro* against the promastigote forms of *L. donovani*, the most common species of *Leishmania* found in India. We have observed that nystatin is able to inhibit the growth of parasite within 24 hours of administration with an $IC_{50}$ value of 105.7µg/ml. In a similar study, the *in vitro* antileishmanial activities of liposomal nystatin was compared with free nystatin, some amphotericin B formulations and antimycotic azole drugs such as ketoconazole, fluconazole and itraconazole against *Leishmania braziliensis*, *L. infantum* and *L. tropica*. It was observed that liposomal formulations of nystatin were more effective than free nystatin against the promastigotes of *L. braziliensis* but were less effective against *L. infantum* and *L. tropica* [12]. In another study, nystatin has also showed very potent *in vitro* antileishmanial activity against *L. major* with an $EC_{50}$ value of 9.76 IU/ml [13]. *L. donovani* is the frequent cause of visceral as well as cutaneous leishmaniasis in the endemic areas of India. Most of the patients with visceral leishmaniasis are non-responsive to glucantime [14]. Therefore, this study was focussed on *L. donovani* for the search of some more effective and safer antileishmanial drug as compared to sodium stibogluconate and amphotericin B. In case of natamycin, we have observed $IC_{50}$ value of 80.49 µg/ml which is lower than nystatin and sodium stibogluconate. Ophthalmic suspension of natamycin which has been used in the present study has reported to possess potent anti-fungal activity against *Fusarium* and *Aspergillus* species [15]. This is the first report of antileishmanial activity of natamycin. The antileishmanial activity shown by standard drug sodium stibogluconate was less as compared to the two research drugs. The calculated $IC_{50}$ was 127.9µg/ml which is less than already reported value of 490.00µg/ml [11]. Amphotericin B has shown very potent antileishmanial activity with an $IC_{50}$ value of 18.91µg/ml which is higher as compared to the previously reported values of 0.9, 1.9, 2.8µg/ml for promastigotes of *L. amazonensis*, *L. chagasi* and *L. amazonensis* respectively [16,17]. It may be concluded that the two research drugs natamycin and nystatin used in this study are not as good antileishmanial agents as amphotericin B but their considerable antileishmanial activity and very low toxicity may make these possible competitors of amphotericin B in near future. This may be done by continuous trials of natamycin and nystatin individually, both in combination and in combination with other antileishmanial agents against various species of *Leishmania*.

An earlier *in silico* molecular docking study had suggested that natamycin and nystatin were able to inhibit the essential enzymes involved in the purine and pyrimidine metabolism of *L. donovani* [18]. The findings of the present study are in accordance with the *in silico* study and demonstrates that natamycin and nystatin are more potent antileishmanial agents than sodium stibogluconate, which is the first-line treatment of visceral leishmaniasis all over the world. Further, *in vivo* study is underway to establish the antileishmanial potential of natamycin and nystatin against *L. donovani*.

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