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Research article

IN VITRO ANTILEISHMANIAL ACTIVITY OF *NYCTANTHES ARBOR-TRISTIS*–A MEDICINAL TREE

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ABSTRACT: Medicinal plants have been used as a source of remedies since ancient times in India. Traditional medicine systems consist of large numbers of plants with medicinal and pharmacological importance and hence represent an invaluable reservoir of new bioactive molecules. *Nyctanthes arbor-tristis* is one of the well known medicinal plants commonly known as night-flowering jasmine. Different parts of this plant are used in Indian systems of medicine for various pharmacological actions and has been used for its hepatoprotective, antiviral and antifungal qualities and used in the treatment of various diseases such as sciatica, chronic fever, rheumatism, and internal worm infections. In an attempt to develop new indigenous drugs against leishmaniasis, we have screened aqueous leaf extract of Nyctanthes arbor-tristis and tested in vitro to assess its potential. The present study deals with the assessment of this plant to establish its antileishmanial activity and mode of action for a potent chemotherapeutic agent against Leishmania pathogen. Aqueous extracts showed 100% inhibition in growth at a concentration of 6mg/ml. However at a lower concentration of 0.9 – 1.8 mg/ml, promastigote growth was inhibited by 60-80% with a IC50 of 0.6mg/ml. The action of Nyctanthes arbor-tristis as a chemotherapeutic agent is found to be mediated through inhibition of superoxide dismutase and simultaneous release of toxic superoxide radical. We propose that Nyctanthes arbor-tristis may be considered as a prospective candidate to establish a better line of therapeutic process against visceral leishmaniasis. The results of this study contribute to the promotion of traditional medicine products and are preliminary for the isolation of new natural molecules for the treatment of leishmaniasis.
Key words: Medicinal plant, Leishmania, Nyctanthes arbor-tristis, Superoxide dismutase

INTRODUCTION

Leishmaniasis has been defined by the World Health Organization as a group of diseases that severely affects 12 million people residing in the warm areas of the world (Desjeux, 2001). In most of the cases, patients cannot survive if proper treatment is not provided during development of this sand fly mediated parasitic disease. Several antileishmanial agents have already been reported (Murray, 2001; Marty and Rosenthal, 2002) but none of these proved to be the ultimate choice of drug due to varying degrees of efficacy and toxicity. Among these, pentavalent antimonials although are recognized to be the most useful drug for treatment of visceral leishmaniasis caused by *Leishmania donovani* (Herwaldt and Berman, 1992), discovery of antimony salt resistant pathogenic strains has made the situation worse to treat the patients against these parasites (Sundar, 2001). As a result, there is a need to identify new chemotherapeutic agents for effective therapy of the visceral form of leishmaniasis, commonly abbreviated as kala-azar. In search for new drugs against leishmaniasis, natural products may offer unlimited source of chemical diversity for identification of new drug templates (Fournet and Munoz, 2002). In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries owing to its natural origin and lesser side effects (Report of the seminar on 'Herbal Drugs, 2001). In rural areas, traditional medicine in leishmaniasis largely remained the only source of treatment being handled by the local people without proper scientific information. Government has also appeared to focus more on the potential of herbal issues (The Times of India, 2003). The interest in the plant products, especially medicinal plants or their extracts, surfaced all over the world due to the belief that many herbal medicines are known to be free from side effects. In an attempt to develop new indigenous drugs, we have screened aqueous leaf extract of *Nyctanthes arbortristis* and tested *in vitro* to assess its potential. The present study deals with the assessment of *Nyctanthes arbortristis* to establish its antileishmanial activity and mode of action for a potent chemotherapeutic agent against *Leishmania* pathogen.

MATERIALS AND METHODS

Preparation of leaf extract of *Nyctantheses arbortristis* :

Leaf extract of *Nyctantheses arbortristis* was prepared following the procedure described below :
52 gms of dried leaves of *Nyctantheses arbortristis* was taken in a jar of REMI Mixer Grrinder. 425 ml of distilled water was added to it. The mixture was grinded in the same grinder for 15 minutes at an interval of 2 minutes between two consecutive uses. The extract was filtered with Whatman #1 (125 mm diameter) filter paper. The residue was discarded and filtrate obtained at this stage was evaporated to dryness in a Flux Evapoartor. The yield of the dried powder of *Nyctantheses arbortristis* was 23% (w/v). The powder was stored at 4^0C in a desiccant for use in various experiments.

Parasites

Leishmania donovani strain MHOM / IN /AG / 83 was obtained from Indian kala-azar patient (Ghosh et al., 1985) and maintained by intracardial passage every 8 weeks in Syrian golden hamsters. Promastigotes were obtained by transforming amastigotes isolated from infected spleen (Jaffe et al., 1984) and maintained in Medium – 199 supplemented with 10% fetal calf serum (FCS) *in vitro.*

In vitro growth of *Leishmania donovani* (AG83) promastigotes in presence of *Nyctantheses arbortristis* extract

L. donovani promastigotes (Jiang et al., 1994) were cultured in Medium-199 with or without *Nyctantheses arbortristis* at 22°C. The crude *Nyctantheses arbortristis* powder was dissolved in dimethylsulphoxide (DMSO) at a concentration of 0.1% after being assayed as non-toxic and without inhibitory effects on parasite growth. Crude extract was added to the culture medium (2.0 ml) with different concentrations ranging from 0 to 6mg/ml (serially diluted). Number of motile parasites was counted under microscope to monitor growth status in absence and presence of testing material. Initially, the parasite concentration was 1.27×10^6 cells/ml, and the culture was used in exponential growth phase. The effect of *Nyctantheses arbortristis* against promastigotes was evaluated after 4 days of inoculation, using a Neubauer Haemocytometric chamber and calculating the percentage of growth inhibition by the formula:

$$\%IC = [T_c - T_p] \times 100 / T_c$$

where IC = percentage of growth inhibition for each period of time and for each dose of the tested product

T_c = Number of flagellate protozoa /ml present in the control tubes

T_p = Average number of flagellate protozoa /ml corresponding to the different products tested and their respective doses

Superoxide dismutase (SOD) assay

Activity of SOD was determined by measuring the inhibition of pyrogallol autoxidation rate (Marklund and Marklund, 1974). The assay mixture contained 0.2mM pyrogallol in air equilibrated 50mM Tris – cacodylic acid buffer, pH 8.2, and 1mM diethylenetriaminepentaacetic acid. The rate of autoxidation was obtained by monitoring the increase in absorbance at 420 nm in a Hitachi spectrophotometer, No U2000. SOD has the ability to inhibit the autoxidation and the extent of inhibition is taken as the measure of enzymic activity.
SOD has the ability to inhibit the autoxidation and the extent of inhibition is taken as the measure of the enzyme activity. One unit of SOD was defined as the amount of enzyme which inhibited the pyrogallol autoxidation rate by 50%.

Determination of superoxide radical release

Promastigote lysates were pre-incubated with different concentrations of *Nyctantheses arbortristis* and then release of superoxide radical was measured spectrophotometrically through the formation of blue formazan (Yasuka, 1978).

SDS-Polyacrylamide gel electrophoresis

SDS-PAGE was performed in 10% polyacrylamide gels.

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Protein estimation

The protein content was determined using Folin Ciocalteu's reagent after precipitating the protein with 2% sodium deoxycholate and 24% trichloroacetic acid. BSA was used as standard.

Statistical analysis

Statistical analyses were conducted through Student's t-test as described (Mishra and Mishra, 1983).

RESULTS

Effect of Nyctantheses arbortristis on *in vitro* growth of *Leishmania donovani* promastigotes

Leishmania donovani promastigotes were cultured individually in vitro upto 4 days at 22°C in presence of different concentrations of *Nyctantheses arbortristis* dissolved in DMSO. Final concentration of DMSO was maintained at 0.1% (v/v). At this concentration DMSO has no effect on the growth of *L. donovani* promastigotes.

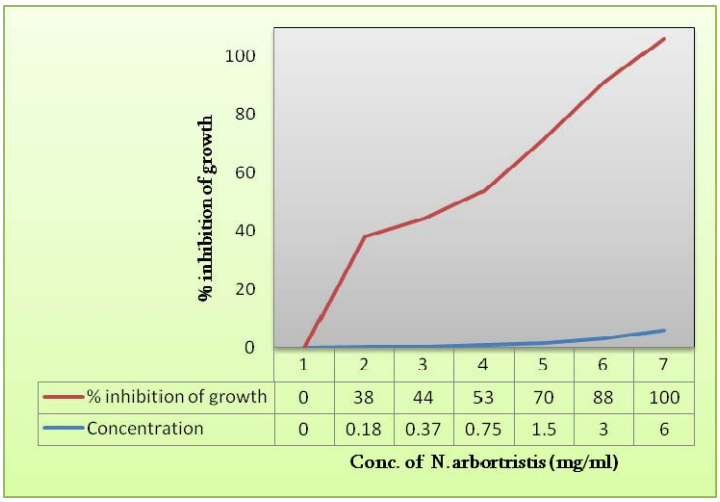

Figure 1: Effect of *Nyctantheses arbortristis* on the growth rate of *Leishmania* promastigotes

At a concentration of 6.0 mg/ml of *Nyctantheses arbortristis*, parasite growth was inhibited by 100% (Fig 1). However at a lower concentration of 0.9 mg/ml to 1.8mg/ml, promastigote growth was inhibited by 60-80%.

Impact of *Nyctantheses arbortristis* on SOD activity and superoxide radical release

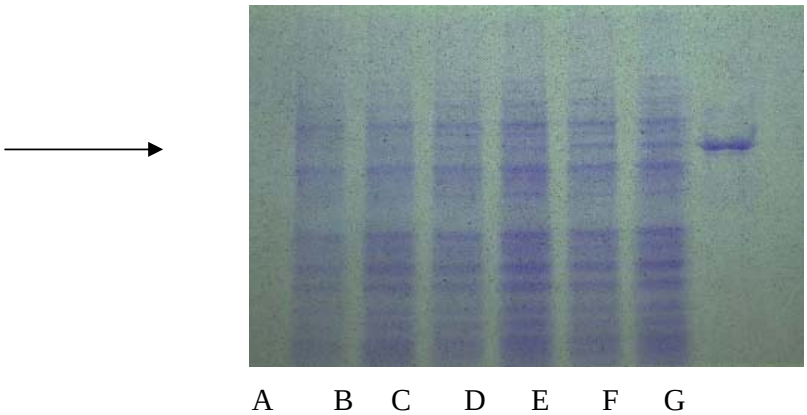

Figure 2 : SDS-PAGE of *Nyctantheses arbortristis treated Leishmania* cell free extract. 60 µg protein was loaded in each lane. (A. 60 mg/ml *N. arbortristis*; B. 40 mg/ml *N. arbortristis*; C. 20 mg/ml *N. arbortristis*; D. 10 mg/ml *N. arbortristis*; E. 5 mg/ml *N. arbortristis*; F. Without *N. arbortristis*; G. pure Fe-SOD).

SDS-PAGE of *Leishmania* cell free extract showed that the activity of *Leishmania* SOD is inhibited with increasing concentration of *Nyctantheses arbortristis.* (Fig 2). No SOD activity was detected at 60mg/ml and 40 mg/ml concentration of *Nyctantheses arbortristis* (Lane A and Lane B – Fig 2).

When promastigote lysate and *Nyctantheses arbortristis* were added simultaneously (Fig 3) in the assay mixture to determine SOD activity, 35.5% inhibition was observed at 20mg/ml. At the same concentration, however, 68.5% inhibition was found when promastigote lysate was pre incubated with *N. arbortristis* prior to SOD assay.

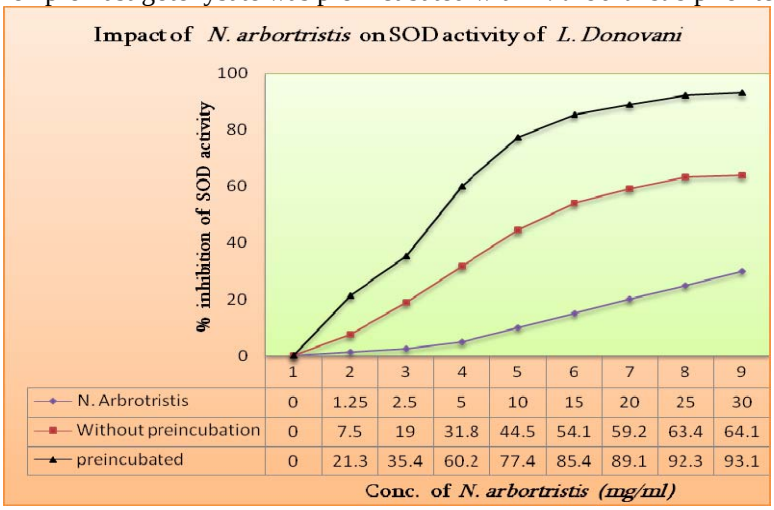

Figure 3: Status of Leishmanial SOD activity (■) when enzyme and *N. arbortristis* were added simultaneously in the reaction mixture or (▲) enzyme and *N. arbortristis* were pre-incubated for 30 min before adding to reaction mixture.

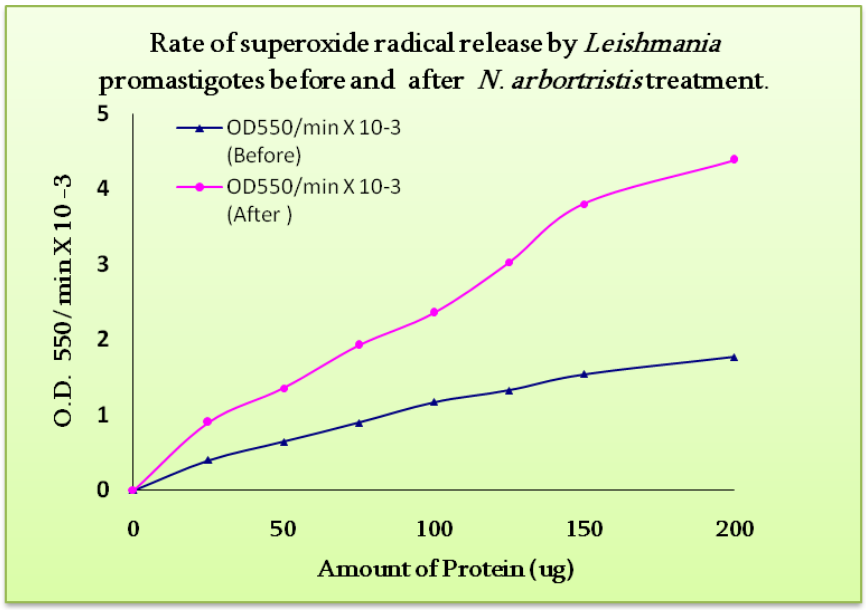

Figure 4: Rate of superoxide radical release by *Leishmania* promastigotes *(▲) before and* (●) *after N. arbortristis* treatment. Formation of blue formazan derived from reduced nitroblue tetrazolium in the presence of superoxide radical was measured spectrophotometrically.

Enhancement in the rate of superoxide radical release was observed (Fig 4) due to *Nyctantheses arbortristis* treatment of *Leishmania* pathogen compared to normal parasite. At 20 mg/ml of *N. arbortristis*, basal superoxide radical decay in parasites increased nearly two fold.

DISCUSSION

Lack of response to sodium stibogluconate is reported in some geographical areas(Pearson, and Queiroz, 1992). In our laboratory a herbal product *Nyctantheses arbortristis* were evaluated for antileishmanial activity *in vitro*. *In vitro* activities were tested on cultured promastigotes of a pathogenic strain of *L. donovani*. *In vitro* studies showed significant antileishmanial activity on cultured promastigotes. The novelty of this extract is that it inhibits 100% parasite growth in culture. The IC_{50} of the synthetic compound is 20 fold less compared to pentamidine. The IC_{50} of *Nyctantheses arbortristis* was found to be 0.6 mg/ml. They may be considered as a good candidate to act as a chemotherapeutic agent against visceral leishmaniasis. These compounds may be suitable alternatives if the antimonial agents are not effective or cannot be tolerated.

Superoxide dismutase (SOD) which is one of the key enzymes of oxygen defense system is known to be an essential factor in mediating normal cellular functions (Fattman et al., 2002). As a result the enzyme has been targeted for the treatment of several diseases (Briedbach et al., 2002). SOD also plays a vital role during host–parasite interaction. Its activity is elevated when *Leishmania* parasite infects host cells (Dey et al., 1995). In our study, it has been found that Leishmanial SOD activity is inhibited during *Nyctantheses arbortristis* treatment to a great extent as shown by SDS-PAGE. Appearance of pure SOD available from a heterologous source (*E. coli*) was found to be also diminished during SDS-PAGE analysis in a dose dependent manner.

At the same time release of superoxide radical which is a microbicidal mechanism of macrophages (Sies and Groot, 1992) were found to be elevated in drug treated cells. Due to deficiency in SOD activity, which is responsible to detoxify released superoxide radicals, toxic free radicals cannot be scavenged up to the maximum limit. In a recent report SOD has been demonstrated to be a key enzyme to play a vital role in the survival of intracellular parasites (Ghosh et al., 2003). Importance of this enzyme in the host–parasite interaction was established by generating SOD-deficient *Leishmania donovani*. It is proposed that the mode of action of *Nyctantheses arbortristis* is mediated through both inhibition of SOD and release of toxic oxygen metabolites for *Leishmania* infection.
The results obtained from different experiments suggest that inhibition of SOD and simultaneous release of superoxide radicals impose toxic effect to destroy intracellular parasites during experimental visceral leishmaniasis. Data suggest that *Nyctantheses arbortristis* may be a better choice to act as an antileishmanial agent with better efficacy and no toxic side effects.

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