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SHORT COMMUNICATION

Nuphar lutea: *In vitro* anti-leishmanial activity against *Leishmania major* promastigotes and amastigotes

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Abstract

Several anti-leishmanial drugs of choice are of plant origin. Many of the available drugs against the disease are toxic and in certain cases parasite drug resistance is developed. The development of new compounds is urgently required.

Aims of the study: To determine the leishmanicidal activity of the *Nuphar lutea* plant extract against *Leishmania major in vitro*.

Materials and methods: The leishmanicidal activity of methanolic plant extract against *L. major* free living promastigotes and intracellular amastigotes was evaluated, using microscopic examinations and the enzymatic XTT assay.

Results: Methanolic extract of *N. lutea* was highly effective against both *Leishmania* promastigotes and *L.* amastigotes ($IC_{50} = 2 \pm 0.12\,\mu g/ml$; $ID_{50} = 0.65 \pm 0.023\,\mu g/ml$; $LD_{50} = 2.1 \pm 0.096\,\mu g/ml$, STI = 3.23). The extract at 1.25 μg/ml totally eliminated the intracellular parasites within 3 days of treatment. Also, a synergistic anti-leishmanial activity was demonstrated with *N. lutea* extract combined with the anti-leishmanial drug – paromomycin. The partially purified *N. lutea* active component was found to be a thermo-stable alkaloid(s) with no electrical charge and is resistant to boiling and to methanol, dichloromethane and xylene treatment.

Conclusions: The present study suggests that *N. lutea* might be a potential source of anti-leishmanial compounds.

Keywords: *Nuphar lutea*; *Leishmania major*; Amastigotes; Promastigotes

Introduction

Leishmaniasis, a zoonotic protozoan disease caused by *Leishmania*, is still considered a major health problem. In the vertebrate host, *Leishmania* are intracellular parasites (amastigotes) of the macrophage of the skin and lymphatic organs, and in the intestine of the invertebrate host (sand fly) they are transformed into free living flagellates (promastigotes). The clinical manifestations range from simple cutaneous lesion (CL) to progressive disseminated visceral (VL) fatal disease. The number of effective drugs available against the disease (sodium stibogluconate, pentamidine, amphotericin B, miltefosine, paromomycin) is extremely limited, and the development of resistant parasites to most drugs has been

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reported (Jha 2006; Santos et al. 2008). In the present study, the anti-leishmanial activity of *N. lutea* extract against *L. major* promastigotes and intracellular amastigotes was evaluated.

Material and methods

Parasite strains, cell cultures and host animals

Male C3H/HeJ, 10–12 weeks old, were used as the source of peritoneal macrophages for *in vitro* studies. *L. major* (WHO code: MHOM/IL/80/Freidlin) was used in all experiments. The parasites were maintained at 28 °C by biweekly passage in RPMI-1640 plus L-glutamine (20 mM), supplemented with 10% heat inactivated FCS and the antibiotics: 100 μg streptomycin and 100 units of penicillin per ml. Parasites were also maintained as a stabilate at −70 °C and *in vivo* in experimentally infected Balb/c mice. Mice were treated according to the NIH guidelines for Animal Care, using a protocol approved by the Animal Ethical Care Committee of Ben-Gurion University.

Plant collection and extracts preparation

N. lutea (mainly leaves) was collected by Prof. Avi Golan, at various seasons from natural habitats in Israel. Additional samples were collected in the Yarkon-Natural Water Reserve in Central Israel. Immediately after harvesting, the plant material was submerged in liquid nitrogen and afterwards at −80 °C until extracted. For the 50% methanol:water (v/v) extraction, samples (1 g) of frozen plant material were ground in a pre-chilled mortar containing liquid nitrogen. Two ml of 50% methanol/water (v/v) were added, and the slurry was mixed and kept on ice for 15 min. After centrifugation at 11,000 rpm for 5 min at room temperature the supernatant was collected and stored at −80 °C for analysis. Larger samples of the plant were further collected, washed with sterile distilled water, freezed dried and afterwards powdered. Concentrations were determined based on dry weight per volume. These samples were further extracted using 2 additional procedures, including: (a) water extraction, boiling at acidic condition, followed by xylene treatment at acidic and basic conditions using a standard procedure. Shortly, grinded powder was first extracted three times with distilled water at 4 °C. The mixture was incubated over 3 days at 4 °C with a gentle mixing using a magnetic stirrer, and then filtered twice through a Whatman-No. 3 filter paper and centrifuged for 10 min at 3000 rpm at 4 °C. The filtrate was acidified to pH 3.0 with 1 M HCl, and after 2 h incubation with gentile mixing at 4 °C it was boiled over 20 min. The resulting precipitate was filtered out and the filtrate was extracted three times with xylene. The aqueous layer was collected and alkalinized (pH-10) with 5 M NaOH and extracted again with xylene. The xylene fraction was collected, acidified with 1 M HCl and after gentle mixing the aqueous layer containing the alkaloids was collected, neutralized by NaOH and concentrated by freeze drying. (b) Methanolic extract was fractionated on a silica gel column using chloroform: ethyl-acetate: diethyl-amine 20:1:1 (v:v:v), as eluant. Column fractions were collected and assayed for anti-leishmanial activity.

Anti-leishmanial efficacy

The anti-leishmanial activity against promastigotes, in culture at 28 °C and amastigotes in C3H mouse macrophages at 37 °C, was determined. Parasites and macrophages were cultivated in RPMI medium containing 10% FCS plus antibiotics. Logarithmic dilutions followed by serial drug dilutions of each extract were tested. RPMI 1640 medium, fetal calf serum (FCS), the antibiotics mixture of gentamycin/streptomycin and glutamine were purchased from Biological Industries (Beit Haemek, Israel). Controls samples included: (a) Growth medium without extracts (100% viability). (b) Appropriate solvent, at the highest concentration to which cells were exposed with the extracts. (c) Positive control, containing the anti-leishmanial drug, paromomycin sulfate (PR) (Farmitalia, Italy).

Effect on *L. major* promastigotes *in vitro*, at 28 °C

Activity of plant extracts against promastigotes was determined by both microscopic counting and the colorimetric cell viability XTT (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (Sigma Chemcal Co; St. Louis, MO) assay (Williams et al. 2003). Promastigotes (2.5×10^6/100 μl/well) from a logarithmic growth phase culture in complete medium were seeded into a flat-bottomed 96-well plastic tissue-cultured microplates, in triplicates, and either logarithmic or serial dilutions of each extract (100 μl) was added. After 3 days incubation at 28 °C, 25 μl of XTT (0.2 mg/ml) were added to each well, followed by an additional 3–5 h incubation at 37 °C. The optimal density (OD) at 450/650 nm was measured using an ELISA plate reader (Dynatech MR5000, USA). The 50% lethal dose (IC_{50}) was evaluated graphically by plotting concentration versus percentage growth inhibition. The anti-leishmanial activity was further determined by microscopic counting of the free living parasites for each well and the percentage growth inhibition for each concentration of extract was calculated in relation to control.

Effect on intracellular *L. major* amastigotes *in vitro*, at 37 °C

The efficacy of plant extracts on *Leishmania* amastigotes was determined as previously described by us (El-On et al. 2007). Briefly, C3H mouse peritoneal macrophages (PM) were prepared from thioglycollate-stimulated mice. Before adding the PM to the plates, 10×10-mm sterile cover slips were placed in the wells of a 24-well (16 mm in diameter) microplate (Greiner, Srikenhawen, Germany). The cell concentration was brought to 6×10^5 cells/ml and 0.5 ml was placed in each well. After 2 h incubation at 37 °C under a 5% CO_2 atmosphere, the medium was replaced by 1 ml fresh complete medium containing 1.5×10^6 promastigotes. After an additional 24 h, the cells were washed twice and then fresh medium was added containing the corresponding concentration of extract. The development of the parasites was checked on the third day of treatment, by the examination of three cover slips for each treatment, after staining with Giemsa stain. The percentage growth inhibition [100%−PS (%)] of each extract on the intracellular amastigotes and the ID_{50} (the concentration inhibiting amastigotes growth by 50%) were evaluated. The parasite survival (PS) indicates the total number of intracellular amastigotes in 400 cells of the treated sample vs. the untreated control and was calculated by applying the expression:

$$PS(\%) = \frac{\text{Total number of intracellular amastigotes (Experimental)}}{\text{Total number of intracellular amastigotes (Control)}} \times 100$$

The isobologram method was used to evaluate the nature of interaction of the *N. lutea* extract and PR as described previously (El-On et al. 2007).

Toxicity to macrophages

The toxicity to macrophages was determined microscopically prior to and after staining with Giemsa and by the XTT enzymatic assay (Williams et al. 2003). In the XTT assay, the peritoneal macrophages (2×10^5/100 µl/well, in a 96 wells microplate) were exposed to various extract concentrations as described above. On the 3rd day of treatment 25 µl of XTT (0.2 mg/ml) were added to each well, and after 3–5 h incubation at 37 °C, the optical density (OD) at 450/650 nm was determined. The 50% lethal dose (LD_{50}) and the therapeutic selectivity index ($TSI = LD_{50}/ID_{50}$), indicating the ratio of toxicity to macrophage vs. toxicity to the parasites, within 3 days of treatment were evaluated.

Results

Up to 1% methanol in complete medium had no toxic effect on either promastigote or amastigote development. *N. lutea* extract was highly effective against *Leishmania* amastigotes ($IC_{50} = 2 \pm 0.12$ µg/ml; $ID_{50} = 0.65 \pm 0.023$ µg/ml; $LD_{50} = 2.1 \pm 0.096$ µg/ml, STI = 3.23). *N. lutea* extract at 1.25 µg/ml totally eliminated the intracellular amastigotes within 3 days of treatment. It was almost as effective as paromomycin ($IC_{50} = 75$ µg/ml, $ID_{50} = 40$ µg/ml, $LD_{50} = 4000$) – the gold standard drug against the disease. However, approximately 56 folds of higher concentration of PR (pure material) compared to *N. lutea* extract (crude material) was required to achieve parasite elimination. The selectivity therapeutic index (STI = 3.23) of *N. lutea* extract was low, compared to STI = 100 obtained with PR. PR at 7000 µg/ml totally destroyed the uninfected macrophages within 3 days of treatment.

Both *N. lutea* extract at 1.25 µg/ml and PR at 70 µg/ml, added to the macrophage culture, totally eliminated the intracellular amastigotes on the 3rd day of treatment. These levels of growth inhibition determined whether the interaction between the two compounds exhibited a synergistic or additive anti-lieshmanial effect. Isobologram analysis indicated that the combination was synergistic across all dose levels tested [*N. lutea*:PR (µg/ml): 0.1:60; 0.25:45; 0.4:35; 0.5:30; 0.7:20; 1:10] with all of the points lying under the line with the additivity (Fig. 1).

Approximately 3 times fold of *N. lutea* extract was required to induce a similar activity against promastigotes ($IC_{50} = 2$ µg/ml) vs. amastigotes ($ID_{50} = 0.65$ µg/ml). Also a direct correlation was observed between the microscopic counting of the promastigotes and the treated uninfected macrophages and the XTT assay.

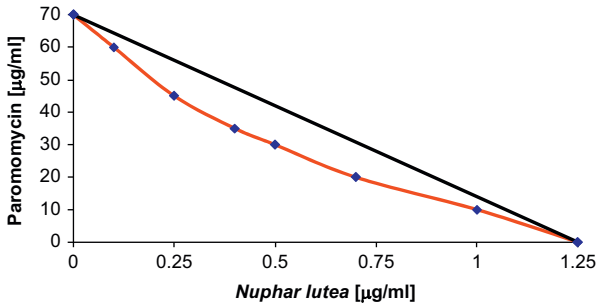

Fig. 1. Isobologram describing synergistic action of *N. lutea* extract and paromomycin acting alone and simultaneously on *L. major* amastigotes in C3H mouse macrophages, on the 3rd day of treatment at 37 °C. *N. lutea* at 1.25 µg/ml and paromomycin at 70 µg/ml administered alone to the culture totally eliminated the parasites. The points illustrate the 100% growth inhibition when *N. lutea* and paromomycin were used in combination.

An IC_{50} value of 50 μg/ml was obtained with the reference drug – PR. This drug at 150 μg/ml totally destroyed the promastigotes within 3 days of exposure.

Further results obtained indicated that both the 50% methanol and water extractions of *N. lutea* are equally effective in the extraction of the active compound. Furthermore, water extraction, boiling at acidic condition, followed by xylene treatment at acidic and basic conditions was highly effective in the extraction of the active compound ($IC_{50} = 0.02 \pm 0.001$ μg/ml; $ID_{50} = 0.061 \pm 0.018$ μg/ml; $LD_{50} = 0.24 \pm 0.014$ μg/ml, STI = 3.93). Fractionation of the *N. lutea* crude extract on a Silica gel column yielded a highly active fraction against *Leishmania* promastigotes ($IC_{50} = 0.033 \pm 0.002$ μg/ml) and amastigotes ($ID_{50} = 0.087 \pm 0.003$ μg/ml) with STI = 3.33. Similar to the crude extract, the semi-purified compound at 0.17 μg/ml totally eliminated the intracellular amastigotes. Preliminary characterization of the partially purified *N. lutea* active component indicated that it is a thermo-stable alkaloid(s) with no electrical charge. It is resistant to boiling and to methanol, dichloromethane and xylene treatment.

Discussion

During the last decade numerous systematic studies from various parts of the world on the anti-protozoal activity of medicinal plants have been reported (Rocha et al. 2005; Braga et al. 2007; Subeki et al. 2007). In the present study, *N. lutea* showed potent inhibitory effect against both promastigotes and amastigotes, and total elimination of the intracellular amastigotes was achieved within 3 days treatment. Indeed, the TSI value (TSI = 3.23) obtained with *N. lutea* was low compared to ~100 observed with PR. However, approximately a 56 folds higher concentration of PR (pure material) compared to *N. lutea* extract (crude material) was required to achieve total amastigotes elimination within the host cell – the macrophage.

Until recently, *Leishmania* drug resistant was demonstrated with most of the available drugs against the disease (Prez-Victoria et al. 2003; Jha 2006). Combined chemotherapy of sodium stibogluconate and PR was very effective, curing approximately 95% of the patients suffering from *L. donovani* infection (Sundar and Chatterjee 2006). In this study *N. lutea* extract was found to act synergistically with PR against *L. major*. It is therefore possible that the purified active compound of *N. lutea* will be found useful either alone or in combination with PR in the treatment of the disease.

Several investigators have reported on the different biological activities of *N. lutea* in various *in vitro* and *in vivo* test models. Extracts of this plant have been found to exhibit anti-cancer (Matsuda et al. 2006; Golan-Goldhirsh et al. 2008) and anti-plasmodial (*P. falciparum*) activity (Sathiyamoorthy et al. 1999). The active compound (s) of *N. lutea* was found to be a mixture of alkaloids with the major active compound being 6-hydroxythionuphlutine B (Golan-Goldhirsh et al. 2008). It is therefore possible that the anti-leishmanial activity of *N. lutea* is mediated by similar components. Nevertheless, further studies are required to be performed in order to characterize the active component of *N. lutea* against *Leishmania*, using highly purified compounds. The results described in this study provide an additional contribution towards the development of new anti-leishmanial compounds.

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