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Journal of Microbiological Methods 55 (2003) 813–816
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Note

Hydrosoluble formazan XTT: its application to natural products drug discovery for *Leishmania*

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Received 28 January 2003; received in revised form 31 July 2003; accepted 14 August 2003

Abstract

A colorimetric method for measuring the viability of *Leishmania* promastigotes is described that is based on the reduction of the tetrazolium salt, XTT, to a water-soluble formazan. Values obtained by the XTT method correlated well with parasite number ($r = 0.965$) and with methods that rely upon the reduction of MTT or MTS ($r = 0.96$ and 0.97, respectively). The IC_{50} values obtained by XTT method with amphotericin-B, miltefosine and ketoconazole were similar to those previously reported by other methods. The XTT method proved to be a reliable and convenient method for the screening of methanolic extracts from 1059 plants and was used for the bioassay-guided fractionation of the alkaloid aegeline from *Sarcorhachis naranjoana*.

Keywords: XTT; *Leishmania*; Natural products; Drug discovery; Aegeline; *Sarcorhachis naranjoana*

Leishmaniasis is a parasitic disease of great epidemiological impact caused by multiple species of the protozoan parasite *Leishmania* (Croft and Yardley, 2002). Its treatment with antimonials, amphotericin-B or pentamidine is associated with multiple side effects making the search for new treatments imperative. Natural products have been a rich source of compounds with antileishmanial activity (Chan-Bacab and Pena-Rodriguez, 2001), revealing the need for an efficient and quantitative method for screening large numbers of extracts and compounds for anti-parasitic activity. Incorporation of labeled [^{3}H]thymidine into the DNA of the multiplying parasite is a reliable assay (Berg et al., 1994), but the use and disposal of radioactive isotopes can be inconvenient or impossible, especially in laboratories based in unindustrialized countries. A colorimetric method based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial dehydrogenase of the parasite is widely used for *Leishmania* growth and shows a high correlation with the radioactive method and with a technique that

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doi:10.1016/j.mimet.2003.08.013

employs the water soluble formazan, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2*H*-tetrazolium (MTS) (Berg et al., 1994). However, the formazan product derived from the reduction of MTT is insoluble in aqueous media, requiring aspiration of the media and the addition of organic solvents in order to read optical density values (OD). The aspiration and solubilization steps are time-consuming, and the latter may introduce error by the accidental removal of parasites from the medium. To avoid this problem, MTT analogs such as sodium-2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide (XTT) and more recently MTS, have been effectively employed in cell proliferation assays. The reduced form of these agents is a water-soluble formazan, which obviates the need for solubilization (Weislow et al., 1989). An additional advantage of the XTT and MTS methods is the shorter period of incubation which results from the addition of the electron coupling agent PMS to accelerate the reduction of the substrate (Buttke et al., 1999). In the present work, the use of XTT in a *Leishmania* promastigote assay is presented and applied to the isolation of natural products with antileishmanial activity.

Experiments were performed with stationary phase *Leishmania mexicana* (WHO-MOHM/B2/82/BELZ) promastigotes, cultured by modification of a previously described method (Morel, 1984). We first determined the correlation between parasite growth curves obtained by manually counting the parasites and by measuring OD values of the formazan product obtained upon reduction of XTT. In this experiment, 250 parasites per well were seeded in two 96-well plates and cultured for 6 days. Three replicates were processed with XTT substrate every 24 h, as described previously (Weislow et al., 1989). Briefly, 50 μl of PBS containing 1.48 mM XTT activated with 200 μM phenazine methosulfate (PMS, Sigma) was added daily. After a 3-h incubation period, OD values were determined in a Benchmark plate reader (BIO-RAD) employing a 450-nm test wavelength and a 650-nm reference filter. Simultaneously, parasites from three wells were counted manually every day, employing a Neubauer chamber. As illustrated in Fig. 1, a strong correlation between both methods was observed ($r = 0.965$).

For measuring the proliferation of *Leishmania* promastigotes, Berg et al. (1994) had previously shown a correlation between [^{3}H]thymidine uptake and the reduction of MTT and MTS. Accordingly, we compared the ability of *L. mexicana* to reduce MTT, MTS and XTT since these substrates are all enzymatically reduced by mitochondrial dehydrogenase (Buttke et al., 1999). The CellTiter 96® aqueous kit, employed for the MTS-based assay (Berg et al., 1994), was also used in the present study (Technical Bulletin #169, Promega). In this experiment, three identical 96-well plates with increasing number of *L. mexicana* were seeded in triplicate and were exposed to MTT, XTT or MTS. While the bioassay employing MTT required 7 h for reduction of the substrate, the methods employing XTT and MTS required only 2 h. Strong correlation was observed between the XTT-based methodology and the techniques employing MTT ($r = 0.96$) and MTS ($r = 0.97$) (Fig. 2).

We also tested the sensitivity of the parasite to antileishmaniasis drugs using the XTT methodology and compared those results to data reported previously. Antileishmanial activity is expressed as IC_{50}, which is a measure of the concentration of test substance that inhibits 50% of parasite growth. The IC_{50} values obtained with ketoconazole were 26 ng/ml, 13 μg/ml for miltefosine and 80–100 ng/ml for amphotericin B, results which are comparable to published values for these compounds (Berman et al., 1984; Kuhlencord et

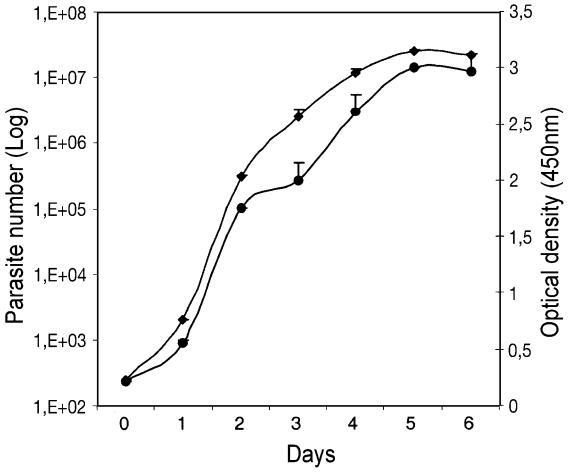

Fig. 1. Comparison of number of *L. mexicana* promastigotes determined by microscopic counting (♦) and from the XTT method (●).

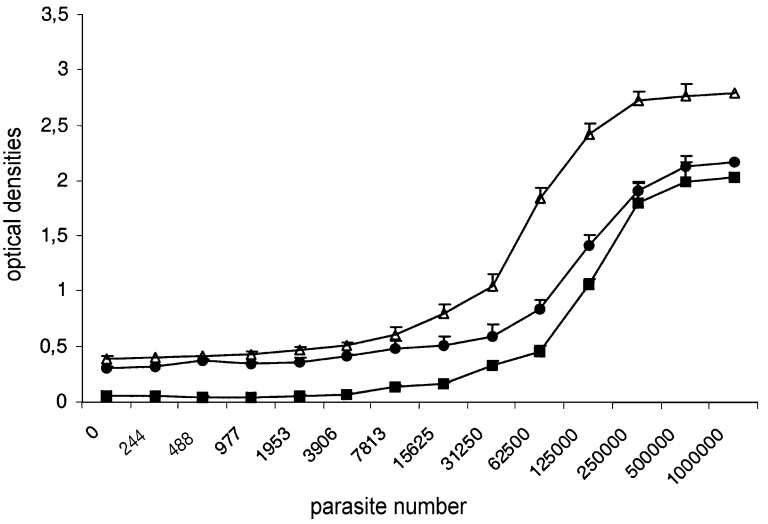

Fig. 2. Comparison of optical density values determined from the reduction of XTT (△), MTS (●) and MTT (■) by *L. mexicana* promastigotes.

al., 1992; Golenser et al., 1999, respectively). The results presented above and in Figs. 1 and 2 indicate the reliability of the XTT methodology as a proliferation assay for *Leishmania* promastigotes. The data presented in Fig. 2 also confirm the reliability of the MTS-based methodology as previously shown by Berg et al. (1994).

The XTT-based methodology is performed in 96-well plates allowing the efficient screening of large numbers of samples. The methodology was used to screen 1059 crude plant extracts of which 8% showed high activity ($IC_{50} < 40$ μg/ml). Plant extracts were prepared following standard protocols (Montenegro et al., 2003). Samples were tested at 300, 42 and 6 μg/ml, in triplicate. Plates were incubated at 26 °C for 72 h in the presence of 5×10^3 parasites in 200 μl of liver infusion tryptose (LIT) medium. At the end of the incubation period, cultures were processed with XTT as described above. As a negative control, parasite growth in the absence of any natural product or drug was measured. To evaluate sensitivity, uniformity and consistency of the parasite response, amphotericin-B was used at concentrations of 1024, 172 and 24 ng/ml. Data analyses were performed with a Microsoft Excel 2000 program, which executed the following operations: (a) calculation of the mean value of the three replicates per sample condition; (b) subtraction of the contribution to the OD value due to the color of the plant extract for each dilution; (c) conversion of the mean OD value to the percentage of viable parasites, where 100% represents the OD of the extract-free controls; and (d) conversion of the percentage of viable parasites to the corresponding IC_{50} value by log-regression.

The XTT methodology has been used for bioassay-guided fractionation yielding biologically active compounds with IC_{50} values ranging from 1 to 5 μM (Montenegro et al., 2003). In the case of the plant, *Sarcorhachis naranjoana* (Piperaceae), the extract was subjected to a liquid–liquid solvent partition with water, methanol, methylene chloride and hexane fol-

Table 1
Antileishmanial activity of samples from the plant *S. naranjoana*, including the crude extract, fractions from purification and purified compounds

Sample	IC_{50} (μg/ml)
Crude extract	15
Water	126
Methanol	67
Hexane	5
Ethyl acetate	93
Aegeline	3.5

lowing literature protocols (Montenegro et al., 2003). The material derived from the hexane partition demonstrated the highest antileishmanial activity and was subjected to silica gel chromatography, yielding the compound aegeline (Sharma et al., 1981), a compound whose anti-leishmanial properties were not previously recognized ($IC_{50} = 3.5$ μg/ml) (Table 1).

In summary, the XTT-based methodology described herein is a straightforward, reliable, efficient and inexpensive means of determining antileishmanial properties in crude biological materials and purified chemical substances. The method should find broad applicability for drug discovery for leishmaniasis, in particular in those areas where the use of radioactive substrates is not an option.

Acknowledgements

The authors wish to acknowledge Dr. María V. Heller for coordinating plant collections, Rafael Aizprua, Blanca Araúz and Nayda Flores for their excellent botanical work and Professor Mireya Correa for plant identification. We thank Nivia Rios and Jorge Aparicio for extract preparation and bioassays, respectively. We thank the Republic of Panama's National Authority of the Environment for facilitating plant collections. We thank Dr. Gordon Cragg of the US National Cancer Institute for the generous gift of XTT. The authors extend special thanks to Drs. Phyllis D. Coley and Thomas A. Kursar who were instrumental in establishing the present bioprospecting program in Panama. This study was supported by an International Cooperative Biodiversity Groups grant, "Ecologically guided bioprospecting in Panama", 5-U01-TW01021-05 from the US National Institutes of Health, the US National Science Foundation and US Department of Agriculture to the Smithsonian Tropical Research Institute.

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