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# THE TRYPANOCIDAL EFFECT OF SESQUITERPENE LACTONES HELENALIN AND MEXICANIN ON CULTURED EPIMASTIGOTES

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ABSTRACT: Sesquiterpene lactones constitute a large group of biologically active compounds obtained from plants. The lactones, mexicanin (MXN) and helenalin (HLN), were reported recently as active against the infective form of *Trypanosoma cruzi*. In this work, we studied the effects of these compounds on the growth and viability of the noninfective epimastigote, to compare the sensitivity of the 2 stages and to characterize their actions. Both compounds were cytotoxic to the parasites, with HLN (inhibitory concentration 50% [$IC_{50}$] 1.9 ± 0.08 μM) more potent than MXN ($IC_{50}$ 3.8 ± 0.19 μM) and the typanocidal drug, benznidazole ($IC_{50}$ 8.6 ± 2.5 μM). The results showed that epimastigotes are less sensitive than trypomastigotes to the compounds. The trypanocidal effect of these lactones, irreversible after 12-hr exposure, was not reversed by the reducing agents dithiotreitol or β-mercaptoethanol. Ultrastructurally, we observed cytoplasmic vacuolization and nuclear disorganization. Although concentrations between 0.5 and 1.5 μM of the drugs were not lethal to the parasites, epimastigotes became thinner and their nuclei became more pycnotic after exposure. We conclude that MXN and HLN are deleterious for *T. cruzi* epimastigotes and that their mechanism of action is different than that of the related lactone, dehydroleucodine.

Natural products obtained from plant leaves are known to have biological activities that have been translated into use as therapeutic agents. Some of these compounds show activity against *Trypanosoma cruzi*, the etiological agent of Chagas disease (Sepulveda-Boza and Cassels, 1996; Bastos et al., 1999; Stoppani, 1999; Brengio et al., 2000; Mafezoli et al., 2000). When cultured, these parasites mostly cycle between the flagellate epimastigote and the nonflagellate amastigote forms. In most cultures, a very low percentage of the parasites differentiate to the infective trypomastigote form in a process termed metacyclogenesis (Isola et al., 1981; Contreras et al., 1994).

Several synthetic and natural trypanocidal compounds have been evaluated, and results with most of them point to the high sensitivity of the parasites to oxidative stress (Penketh et al., 1987; Morello, 1988). However, the high cytotoxicity of these compounds for host cells has prevented their therapeutic use. Plant metabolites active against *T. cruzi* have been used as trypanocidal agents, including the hydroquinone derivative miconidin (Brener, 1973) and some monoterpenes (Kirchhoff, 1993; Sepulveda-Boza and Cassels, 1996). Among the natural products with potential to be used as trypanocidal agents, sesquiterpene lactones appear to be attractive because of their abundance within plant leaves and because of their potent biological activities as antimicrobial and antitumoral agents (Kupchan et al., 1971; Rodriguez et al., 1976; Robles et al., 1995). Some sesquiterpene lactones have already been assayed for activity against *T. cruzi* (Sepulveda-Boza and Cassels, 1996; Bastos et al., 1999; Stoppani, 1999; Brengio et al., 2000; Mafezoli et al., 2000). The electrophilic sesquiterpene lactone, 15-deoxygoyazensolide, is able to destroy the parasites, although this effect is observed only at fairly high concentrations (Chiari et al., 1991). Recently, 2 sesquiterpene lactones, mexicanin (MXN) and helenalin (HLN), were found to be highly toxic to trypomastigotes of *T. cruzi* (Schmidt et al., 2002).

In this work, we extended the study of Schmidt et al. (2002) to the effects of these lactones on the growth, viability, and morphology of epimastigotes to characterize the action of these drugs. Furthermore, we compared their effects with those of the related lactone, dehydroleucodine (DhL).

## MATERIALS AND METHODS

### Cell cultures

Parasites (Tulahuen strain) in the proliferative stage were cultured in Diamond liquid medium (0.106 M NaCl, 29 mM $KH_2PO_4$, 23 mM $K_2HPO_4$, 12.5 g/L tryptose, 12.5 g/L tryptone, and 12.5 g/L yeast extract, adjusted to pH 7.2) supplemented with 2% fetal bovine serum. Parasites ($0.8 \times 10^6$/ml) were added to each tube in the presence or absence of the lactones (at final concentrations of 0.5–9.6 μM) and incubated at 29 C for 3–4 days. Aliquots of the parasites, collected every 24 hr, were either tested for viability by trypan blue dye exclusion or fixed with 10% formalin and counted in a neubauer hemocytometer. Aliquots of each sample were also processed for electron or light microscopy.

Mouse embryonic fibroblasts were kindly provided by Dr. R. Pohlmann (Münster, Germany) and grown in Dulbecco minimal essential medium, supplemented with 10% fetal calf serum. These cells were used as controls to compare cytotoxicity.

### Electron and light microscopy

Parasites were fixed and processed as described (Brengio et al., 2000). In brief, samples were fixed in 3% glutaraldehyde for 24 hr, washed 3 times with 10 mM phosphate-buffered saline (PBS), pH 7.2, and postfixed in 2% $OsO_4$ overnight. The cells were then washed once with PBS and stained with 1% uranyl acetate. They were then dehydrated in graded ethanol and acetone and embedded in Epon 812. Ultrathin sections were cut with an automatic Leica-ultracut Rultramicrotome and observed with a Siemens Elmiskop I. For light microscopy, the parasites were fixed in methanol and stained with Giemsa.

### Extraction and purification of MXN and HLN

MXN I and HLN were isolated from aerial parts of *Gaillardia megapotamica* (*Spreng.*) *Baker* var. *Radiata* collected in San Luis, Argentina (voucher Del Vitto & Petenatti 2841 deposited in the Herbarium at the Universidad Nacional de San Luis [UNSL] San Luis, Argentina). The air-dried material was soaked in chloroform at room temperature (3 times for 48 hr). The combined chloroform extracts were evaporated under vacuum, dissolved in 95% ethanol, and then 4% aqueous lead tetraacetate solution was added. The cloudy aqueous solution was filtered through a celite pad, and the filtrate was concentrated under vacuum. The mixture was extracted 3 times with chloroform, and the solution was concentrated under vacuum. The residue was chromatographed in a medium-pressure chromatography system. Different proportions of ethyl acetate and hexane were used as eluants. The purity of these compounds (>95% for both lactones) was tested by $^{13}C$-nuclear

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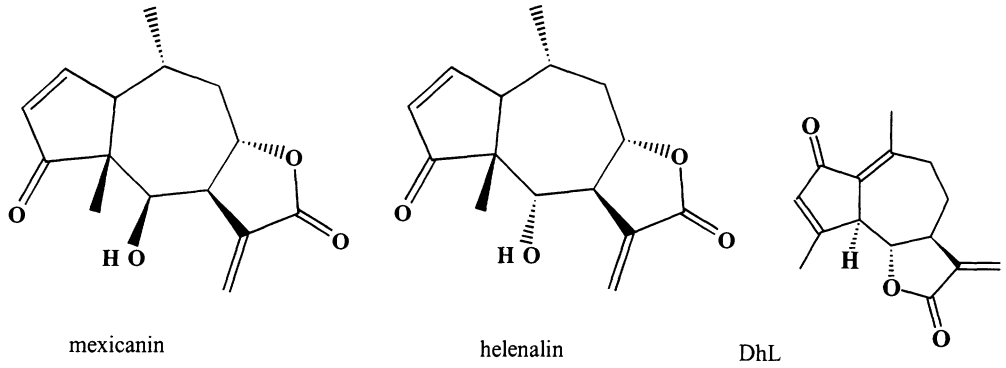


FIGURE 1. Structures of the sesquiterpene lactones. DhL, dehydroleucodine.

magnetic resonance, melting point analysis, and optical rotation, and the data were in agreement with those reported previously (Giordano et al., 1990).

## RESULTS

In this study, we tested the effect of the sesquiterpene lactones, HLN and MXN (Fig. 1), on the growth and viability of cultured epimastigotes of *T. cruzi*. As shown in Figure 2, HLN and MXN were highly active against the parasites because at concentrations ≥1.9 μM growth was stopped within a few hours of treatment. Although both lactones were cytotoxic against this form of *T. cruzi*, HLN was more potent than MXN (inhibitory concentration 50% [$IC_{50}$] 1.9 ± 0.08 and 3.8 ± 0.19 μM, respectively) or the trypanocidal drug benznidazole ($IC_{50}$ 8.6 μM), as determined by the trypan blue dye exclusion test (Fig. 3). Interestingly, these lactones were less cytotoxic for other cell types, such as cultured mouse embryonic fibroblasts ($IC_{50}$ >4 μM). Compared with trypomastigotes (Schmidt et al., 2002), epimastigotes were less sensitive (2.7 times to HLN and 2.1 to MXN) than the infective form.

When compared with the effect of the related lactone DhL, MXN and HLN were more potent against the parasite and certainly appear to act by a different mechanism because the effect of these compounds was not reversed by the reducing agents glutathione (GSH) or dithiothreitol (DTT), as ocurred with DhL (Brengio et al., 2000) (Fig. 4). Because the compounds were dissolved in dimethyl sulfoxide, we tested the effect of the solvent on the growth and viability of the parasites, although no effect was observed (data not shown).

### Changes in parasite morphology

After 24–48 hr of exposure to MXN or HLN, epimastigotes were observed by light and transmission electron microscopy (TEM). Parasites were thinner and exhibited pycnotic nuclei (Fig. 5) in response to MXN or HLN. Although the ultrastructure of the parasites did not show significant changes after 24-hr exposure (data not shown), by 48 hr, cells possessed large vacuoles and exhibited nuclear disorganization, a symptom of the deleterious effect of these compounds (Fig. 6). In both cases, the organization of the cytoskeleton neighboring the plasmalemma was apparently maintained. Interestingly, at concentrations between 0.4 and 0.8 μM, neither HLN nor MXN affected the viability and growth of the parasites, but morphological changes were still observed by light microscopy. The parasites became thinner and their nuclei pycnotic, although

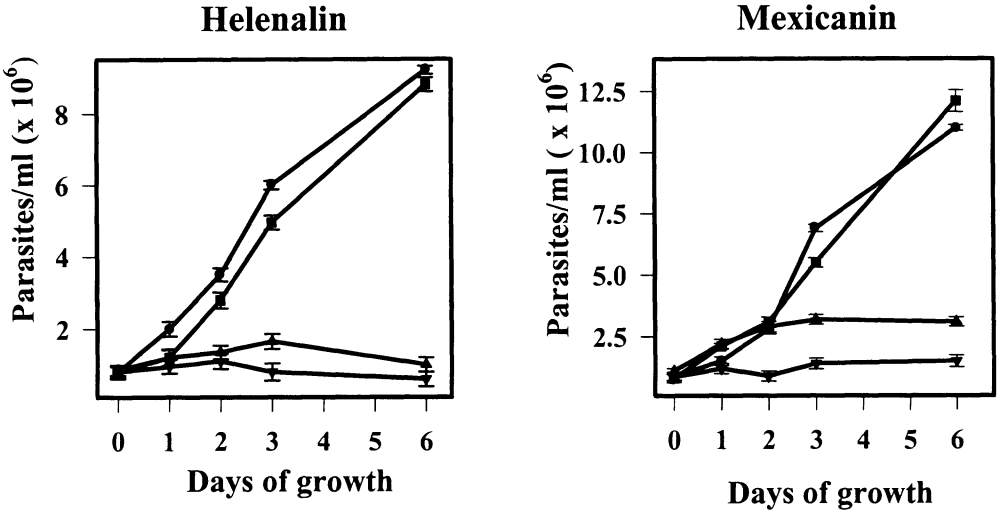


FIGURE 2. The effect of sesquiterpene lactones on the growth of epimastigotes of *Trypanosoma cruzi*. The parasites (1 × $10^6$ cells/ml) were incubated in the absence (●), or in the presence of 0.7 (■), 1.9 (▲), or 3.8 (▼) μM of the corresponding lactone in the medium (see Materials and Methods). The concentration of the parasites was determined at the indicated times. The values represent the mean of 4 experiments (3 replicates per experiment) ± SD.

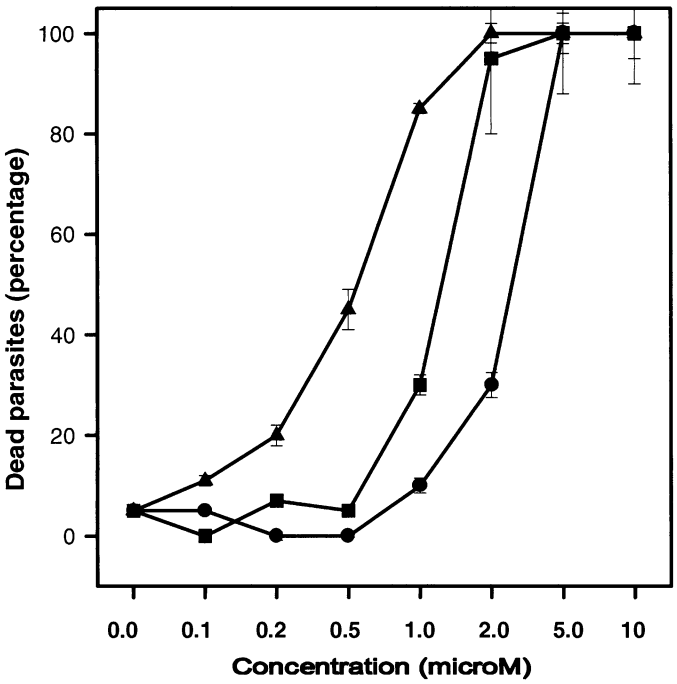


FIGURE 3. The effect of sesquiterpene lactones on the viability of epimastigotes of *Trypanosoma cruzi*. The parasites ($1 \times 10^6$ cells/ml) were incubated for 24 hr in the absence or in the presence of MXN (■), HLN (▲), or benznidazole (●) at the concentrations indicated. Viability was determined by the dye exclusion test (see Materials and Methods). Each point represents the mean percentage of mortality of parasites from 4 experiments (2 replicates per experiment) ± SD.

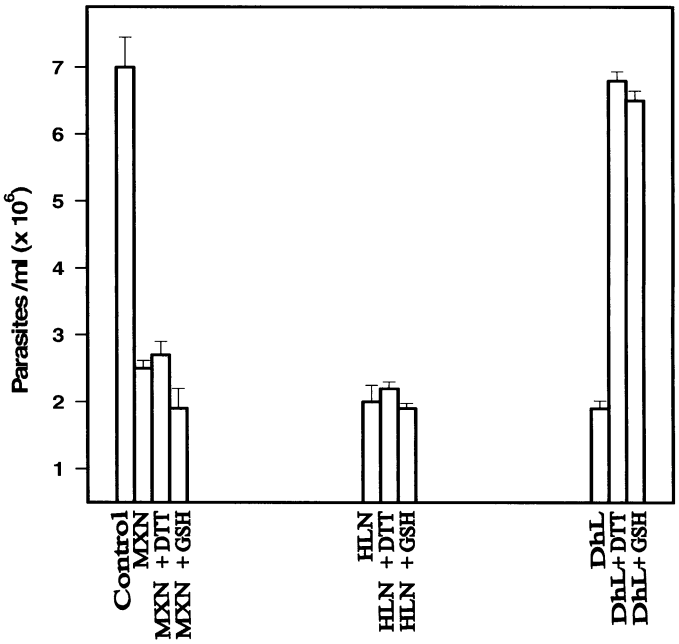


FIGURE 4. Number of parasites on the third day of growth after treatment with either 1.9-μM MXN, 1.9-μM HLN, or 9.6-μM DhL, in the presence or in the absence of either 5-mM GSH or 5-mM DTT as indicated. The values represent the mean of 3 experiments (3 replicates per experiment) ± SD.

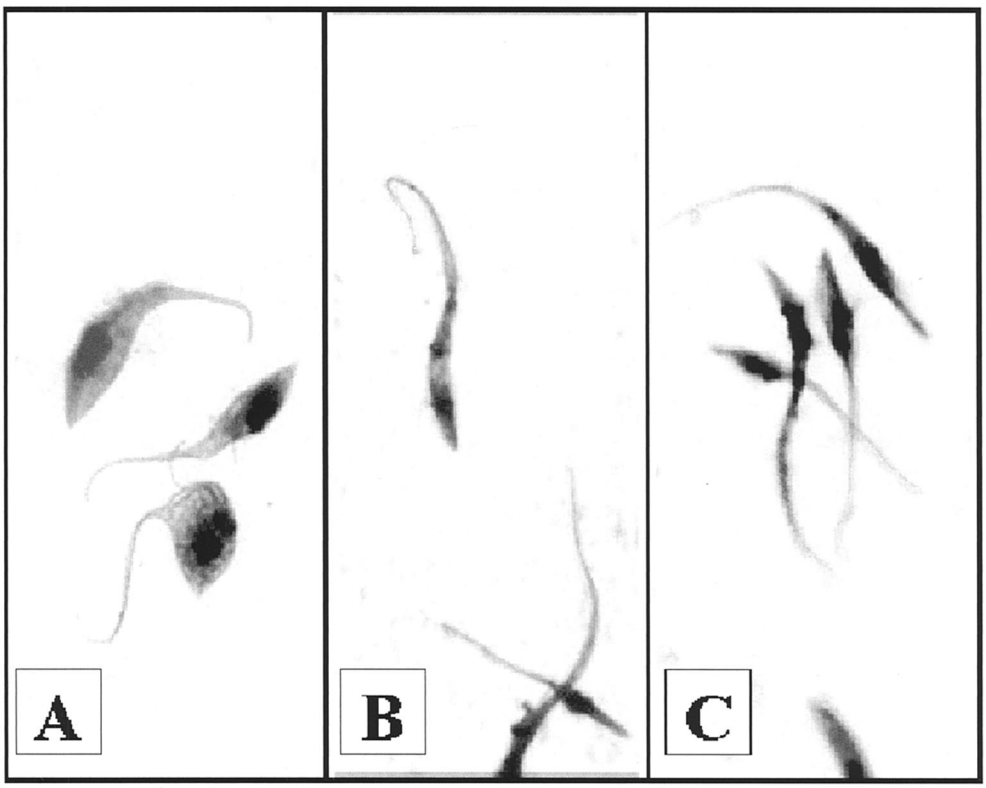


FIGURE 5. Morphology of cultured *Trypanosoma cruzi* at 36 h of growth. Swabs of parasites were stained with Giemsa as described in the Materials and Methods. (**A**) Control. (**B**) Treated with 0.5-μg/ml MXN. (**C**) Treated with 0.5-μg/ml HLN.

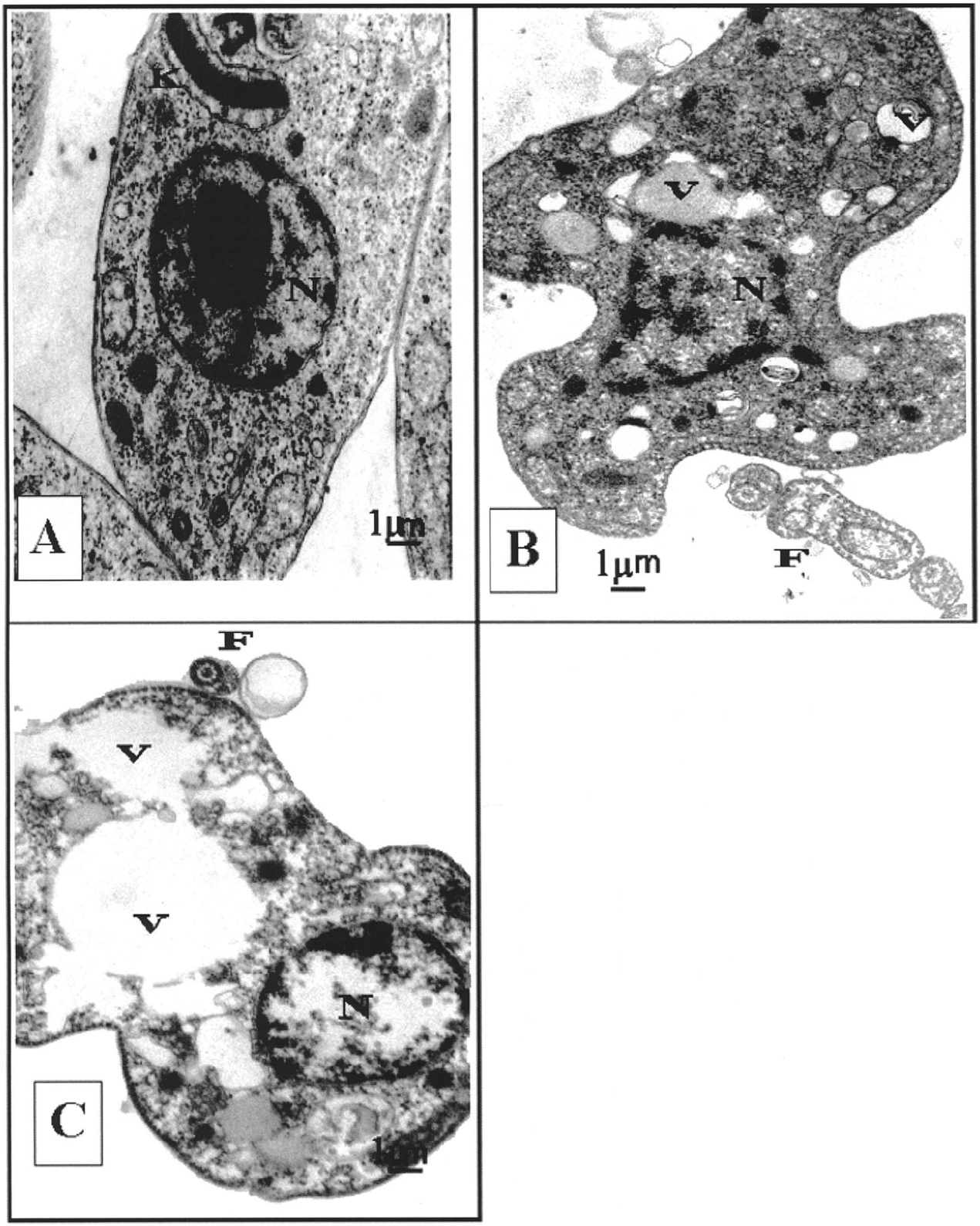


FIGURE 6. Ultrastructure of cultured *Trypanosoma cruzi* on the second day of growth in medium. Parasites were processed as described in Materials and Methods and observed by TEM. (**A**) Control. (**B**) Incubated with 1.9-µM MXN. (**C**) Incubated with 1.9-µM HLN. (N) nucleus, (K) kinetoplast, (F) flagellum, and (V) vacuoles. Magnification: ×5,000.

they did not resemble metacyclic forms. We cannot explain these morphological changes at low concentrations.

## DISCUSSION

In this study, we confirmed the cytotoxic action of MXN and HLN on epimastigotes of *T. cruzi* and showed that this form of the parasite is less sensitive to the drugs than the infective trypomastigote stage (Schmidt et al., 2002).

Although these lactones have antitumor activity, their mechanism of action is poorly understood. It has been reported that HLN increases cyclic adenosine 3′, 5′monophosphate (cAMP) by inhibiting phosphodiesterases, causing $Ca^{2+}$ influx in the myocardium and enhancing contractility (Itoigawa et al., 1987). This may be 1 action of HLN on *T. cruzi* because $Ca^{2+}$ and cAMP levels regulate signal transduction pathways involved in environmental sensing in the parasites (Parsons and Ruben, 2000).

It is known that the α-methylene-γ-lactone moiety of sesquiterpene lactones is responsible for most of the biological properties of these compounds (Giordano et al., 1992). Similarly, some authors suggest that the cytotoxicity of these compounds is mediated by an interaction of the α-methylene with sulphydryl groups of certain enzymes involved in cellular metabolism (Hwang et al., 1996). However, this is not likely to be the mechanism of action of MXN and HLN because neither DTT nor GSH reversed the effect of these compounds. Therefore, we may postulate that either the cyclopentenone group present in MXN and HLN or the spatial orientation of certain groups, e.g., hydroxyls or the γ-lactone itself, might be responsible for the trypanocidal effect of these compounds.

The nuclear disorganization observed by TEM may show an apoptotic effect of the drugs on the parasites. Irreversible damage to parasite DNA by MXN and HLN is conceivable, given that interactions of sesquiterpene lactones with DNA have been reported (Lee et al., 1977). However, we were not able to discern whether this nuclear disorganization preceded other effects, such as vacuolization and changes in morphology, or if all these drastic changes occurred simultaneously. Furthermore, at shorter time points of treatment, e.g., 16 or 30 hr, the ultrastructural characteristics of the cells were not significantly modified (data not shown). MXN and HLN are potential candidates to be used as trypanocidal agents in the fight against Chagas disease and also as tools to elucidate the molecular differences between the infective and noninfective stages that account for differences in sensitivities to the sesquiterpene lactones.

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