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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2004, p. 3828–3833 Vol. 48, No. 10
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.10.3828–3833.2004

Antimicrobial Activity of Euplotin C, the Sesquiterpene Taxonomic Marker from the Marine Ciliate *Euplotes crassus*

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Received 3 November 2003/Returned for modification 9 February 2004/Accepted 23 June 2004

Strains of the marine ciliate protist *Euplotes crassus* produce exclusive terpenoids called euplotins that play an ecological role. Among these derivatives, euplotin C is the main of four secondary metabolites isolated from cultures of this protozoon and represents the sesquiterpene taxonomic marker from *E. crassus*. Because different terpenoid metabolites of plant origin showed a certain antimicrobial activity, we assessed the compound euplotin C, purified by high-pressure liquid chromatography and solubilized in two solubility enhancers, against the protozoa *Leishmania major* and *Leishmani infantum*, the fungus *Candida albicans*, and nine strains of gram-positive and gram-negative microorganisms. An activity of euplotin C against *Leishmania* promastigotes was demonstrated (50% lethal doses were 4.6 or 8.1 μg/ml depending on the agent used to solubilize the compound), while the effect was less evident on *Candida* and nearly absent on bacteria. A nonsignificant cytotoxicity (50% lethal dose, >200 μg/ml) against the J774 cell line was observed. A leishmanicidal activity was also shown by the living, euplotin-producing cells of *E. crassus* cultured together with promastigotes; this activity increased with time from 10 min to 6 h of incubation. This study provides an initial rationale for the evaluation of euplotin C and other similar natural products as alternative or possibly synergistic compounds for current antiprotozoon chemotherapeutics.

In recent years, several reports have been published on the scientifically confirmed antimicrobial activity of some natural products derived from plants (1, 8, 13, 14, 30, 34, 38, 39) or of bioactive metabolites of microbial origin (26, 32, 33, 35, 40). Natural products showing effective action of pharmaceutical and medical relevance have also been recovered from marine organisms. Some bioactive antiviral, antitumor, antimicrobial, or generally cytotoxic compounds have been extracted from species of starfish and sponges. For example, extracts from the starfish *Asterina pectifera* are active against *Aspergillus* spp. and *Cryptococcus neoformans* (7), while various sponges exhibit antibacterial properties which are due to the presence of a metabolic product or to host microorganisms (12, 25).

Recently, four sesquiterpenes—euplotin A, euplotin B, euplotin C (EC), and their biogenetic precursor preuplotin—have been isolated from *Euplotes crassus*, a eukaryotic unicellular marine ciliate (16, 19). All strains of this ciliate morphospecies (taxonomically described by microscopic, outwardly identifying features) produce the same compounds. Notably, euplotins, and in particular EC, are biosynthesized in relative large amounts, suggesting an important ecological role of this substance in the adaptive strategy of the organism. A similar terpenoid (udoteatrial hydrate) was isolated from *Udotea flabellum* (36), a tropical seaweed belonging to the phylum *Chlorophyta* that contributes to the structure of coral reefs. Indeed, it has been suggested that strong similarities in anthozoan metabolite biosynthesis reveal the evolution of terpenoid functions from defensive agents to hormones (41).

Among the different metabolites screened from plants, extracts containing terpenoids, tannins, flavonoids, and naphthoquinones have been demonstrated to have in vitro antimicrobial (9), antiplasmodial (18), and leishmanicidal activity (29, 43).

EC is a lipophilic compound that may represent the final product of the metabolic pathway involving the euplotin family, and it is the most powerful cytotoxic agent among the related sesquiterpenes (euplotin A, euplotin B, and preuplotin) (16, 19).

With the knowledge of these biological effects as our starting point, we searched for a possible in vitro antimicrobial activity of EC. The present study was designed to examine the activity of this compound against nonmarine eukaryotic microorganisms, the pathogenic protozoa *Leishmania major* and *Leishmania infantum* and the opportunistic yeast *Candida albicans*, as well as against some prokaryotic (opportunistic or pathogenic) microorganisms. Moreover, the cytotoxic action of EC against the macrophage-like cell line J774, used as a mammalian host cell control, was assessed.

MATERIALS AND METHODS

EC. *E. crassus* strain SSt22 cells were harvested from mass cultures (grown with the green microalga *Dunaliella salina* as a food organism); they were closely packed and formed pellets upon centrifugation. The culture medium was discarded, because it did not contain any sesquiterpenes. Pellets were extracted with absolute ethanol and filtered. The filtrate was evaporated in a vacuum at room temperature, and the residue was partitioned between hexane–ethyl acetate (8:2) and distilled water, with a relative volume of 2 parts of distilled water to 1 part of organic solvent. The organic extract was then subjected to flash chromatography on a Si60 LiChrolut (Merck, Darmstadt, Germany) by using hexane–ethyl acetate gradient elution, and 17 fractions of 30 ml each were collected. Fractions

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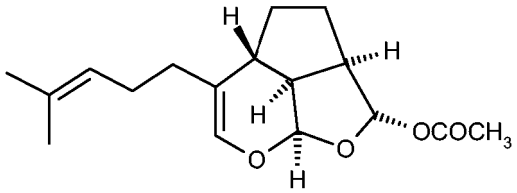

euplotin C

FIG. 1. Chemical structure of EC, the compound used in this study.

8 to 12 were further purified by high-pressure liquid chromatography (HPLC) with a LiChrosphere Si60 (Merck) 5-μm-diameter column (hexane–ethyl acetate, 97/3; UV detector at a λ of 215 nm) to produce pure EC (Fig. 1), whose structure had been previously elucidated (16, 19, 21, 22). A purity control of isolated EC was carried out by mass spectrometry coupled to HPLC via an electrospray interface. Analyses were performed on an Esquire-LC (Bruker Daltonics, Bremen, Germany) ion trap mass spectrometer coupled to an HPLC series 1100 (Hewlett-Packard, Palo Alto, Calif.) equipped with a reversed-phase column (Zorbax Eclipse XDB-C_{18}; 4.6 by 150 mm; Agilent Technologies, Palo Alto, Calif.) with UV absorption monitoring (Photo Diode Array series 1100; Hewlett-Packard) at a wavelength of 215 nm. Approximately 1 mg of pure EC can be obtained from 1 ml of cell pellet. This euplotin, named complex 1, exhibits the typical hydrophobic properties of sesquiterpenes. Hence, suspension in water required addition of 1% dimethyl sulfoxide (DMSO; Merck). Complex 2 was prepared by including euplotin in commercially available heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DIMEB; Sigma-Aldrich, Milan, Italy) by mixing equimolar amounts of EC and DIMEB in a saturated water solution, heating and magnetically stirring for 1 h, sonicating, and allowing the mixture to rest at room temperature for 3 h. The solid-state inclusion complex was obtained by freeze-drying the precipitate separated from the saturated solution (24). In this study we tested the two complexes of EC: complex 1, a pure, colorless oil preparation (1.0 mg/ml) in DMSO and successive dilutions in H_2O, and complex 2, a white powder consisting of an EC-DIMEB (1:1) complex at a concentration of 1.0 mg/ml in H_2O.

***Euplotes* strains.** *E. crassus* strain SSt22, a euplotin-producing and euplotin-resistant strain of the protozoan ciliate, and the closely related morphospecies *Euplotes vannus* strain TB6, a non-euplotin-producing and euplotin-sensitive strain, as reported by Dini et al. (16), were used in the biological assays. These strains have already been used in ecotoxicological studies (16, 42) and were available as part of the personal collection of one of the authors of the present paper (F.D.). These ciliates were cultured at 23 ± 1°C in sterile, defined artificial seawater (Allen's formula) (3) inoculated with the microalga *D. salina* as a food organism (17); in this medium these protozoa divide twice in 24 h.

Leishmaniae. *L. major* (strain LRC-L137) and *L. infantum* (strain MHOM/TN/80/IPT1) were maintained in vitro at 25°C in Tobie's diphasic medium modified according to the work of Taylor and Baker (46). Before use, promastigotes were grown in 199 medium (Invitrogen, Carlsbad, Calif.) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 100 U of penicillin/ml, 100 μg of streptomycin/ml, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 μg of hemin/ml (in 50% triethanolamine), and 1 μg of 6-biotin/ml (in 95% ethanol). In this medium, referred to below as complete medium, the leishmaniae were cultured at 25°C for 5 days to reach the stationary phase of growth (44). Then they were collected by centrifugation (at 1,400 × *g* for 5 min at room temperature), washed in saline solution, and resuspended in fresh complete medium to a final concentration of 2×10^5 viable promastigotes/ml. The number of live organisms was determined by counting with a hemocytometer (Thoma chamber) after vital staining with trypan blue (dye exclusion method).

Fungal and bacterial microorganisms. Organisms tested included one strain of the eukaryotic fungus *C. albicans* (strain 6, a clinical isolate) and nine bacteria, four gram-positive (*Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* ATCC 6538, *Streptococcus pyogenes* ATCC 11434, and *S. pyogenes* ATCC 8668) and five gram-negative (*Escherichia coli* HB101, *Proteus vulgaris* N9, *Pseudomonas aeruginosa* ATCC 15442, *Burkholderia cepacia* ATCC 17759, and *B. cepacia* ATCC 1/2000) clinical and reference strains. These microorganisms were cultured in brain heart infusion (BHI) broth (Oxoid, Milan, Italy).

In vitro leishmanicidal activity. The effects of the two complexes of EC on promastigotes of *L. major* and *L. infantum* were assessed by a method similar to one described previously (44). Promastigotes (2×10^5 viable cells/ml) were incubated in complete medium in the presence of 100, 20, or 2 μg of the preparations/ml. At the same time, controls were prepared in complete medium, in DMSO at the same concentrations used for complex 1 (0.1, 0.02, and 0.002%) and in DIMEB at the same molarity used for complex 2. After 2, 24, or 48 h of incubation at 25°C, parasite survival was estimated by microscopic counting of the number of viable (showing motile behavior and/or lack of staining) promastigotes in the treated cultures. The experiments were performed in triplicate, and the results (means of the triplicate samples at each point) are reported as percent viable leishmaniae relative to the level in the control (measured in medium), which was taken as 100%. At the end of every incubation time, the reversibility of the effect was assessed by adding fresh complete medium at a 10:1 ratio to the cultures. The same dilution was made for the control. After a further 48-h incubation, the number of viable promastigotes was microscopically evaluated. The results are reported as inhibitory and killing activities of the two complexes of EC at different times.

Subsequently, the activity of each of the two different preparations of EC against *L. major* was assessed in twofold serial dilutions from 200 to 3 μg/ml in triplicate on two different days. The 50% lethal dose (LD_{50}, defined as the concentration of the complex which caused a 50% reduction in survival or viability in comparison to that in an identical culture without the compound) was evaluated after 24 h. This value was determined by nonlinear regression analysis, by plotting the number of viable promastigotes versus log EC concentrations by use of GraphPad Prism 3 software.

In vitro antifungal and antibacterial activities. Log-phase cultures of the microorganisms were diluted to 10^5 CFU/ml in BHI broth (Oxoid) and incubated separately for 2 or 24 h at 37°C with one of the two different preparations of EC (complex 1 and complex 2) at 100, 50, or 25 μg/ml by using a technique slightly different from the dilution testing presented in the NCCLS document (37) in order to increase the sensitivity of the test. Another series, using only the organic solvent DMSO or DIMEB to bring EC into solution, was treated identically. After the different observation periods, to assess the killing activity of EC and solvents, 50 μl of the microorganism suspensions was spread onto BHI agar (Oxoid) and incubated for 24 h. CFU of microorganisms were counted and compared with the CFU of controls in medium without EC.

Cytotoxic activity. The cytotoxicities of the two complexes of EC were assessed in the murine monocyte-like cell line J774 (3×10^4 cells/0.1 ml) cultured in complete Dulbecco's modified Eagle medium (Invitrogen) for 24 h at 37°C in a humidified, 5% CO_2 incubator, in 96-well plates (Nunc, Roskilde, Denmark). After removal of the supernatant from the cell monolayer, fresh medium without or with serial dilutions (from 200 to 3 μg/ml) of the EC preparations was added to each well. After 24 h of incubation at 37°C in a humidified 5% CO_2 incubator, cell viability was assessed by spectrophotometric analysis (31) of the cells stained by using Hemacolor reagents (Merck) and an automated microplate reader (Bio-Rad, Milan, Italy). Each experiment was performed in triplicate, and the

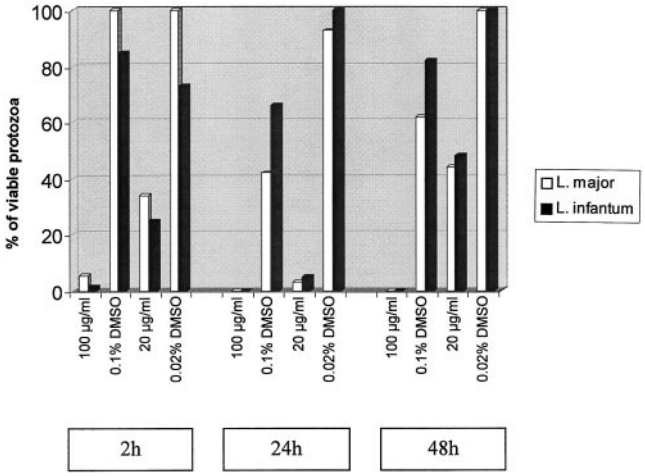

FIG. 2. Inhibitory effects of EC complex 1 (at 100 and 20 μg/ml) and the solvent DMSO (at 0.1 and 0.02%) on the growth of *L. major* and *L. infantum* after 2, 24, and 48 h of contact. Percentages were calculated relative to the growth of promastigotes in medium, considered as 100%.

TABLE 1. Inhibitory and killing activities of EC against *L. major* at different times

EC complex	% Activity[a] of EC at the indicated time and concn against *L. major*											
	After 2 h				After 24 h				After 48 h			
	100 μg/ml		20 μg/ml		100 μg/ml		20 μg/ml		100 μg/ml		20 μg/ml	
	A	B	A	B	A	B	A	B	A	B	A	B
1	94	92	66	60	100	100	97	92	100	100	56	51
2	83	78	42	39	95	92	72	69	100	100	90	85

[a] A, inhibitory activity, B, killing activity.

mean of each point was evaluated. Cytotoxicity data obtained by spectrophotometric analysis were calculated according to the formula devised by Keysari (31), and the LD_{50} was reported. These results were confirmed by microscopic observation of cell monolayers.

In vitro activity against *E. vannus*. A semistarved culture (4×10^4 cells/ml) of the non-euplotin-producing strain TB6 of *E. vannus* was treated with 5- and 10-μg/ml concentrations of the two complexes of EC in sterile, defined artificial seawater. Controls were prepared with seawater and DMSO or DIMEB at the same concentration present in the respective EC complex. The effects of the treatments on the organism were assessed (three times) microscopically by counting the viable (motile) ciliate cells with the aid of a Thoma chamber. Counts were scored throughout an interval ranging from 10 to 180 min at room temperature.

***Euplotes-Leishmania* interaction.** Preliminary experiments using sterile, defined artificial seawater diluted in different ratios with physiological solution (0.9% NaCl) were carried out to establish suitable culture medium conditions for the *Euplotes* and *Leishmania* organisms. The interaction between the two protozoa was then evaluated by using a final cell density of 4×10^4/ml for the two strains of *Euplotes* (*E. crassus* SSt22 and *E. vannus* TB6), separately mixed with a culture (10^6 cells/ml) of *L. major* or *L. infantum*. After 30 and 60 min and after 2- and 6-h incubations at room temperature, the numbers of viable and motile leishmaniae and euplotes were microscopically evaluated by counting with a Thoma chamber. Controls were prepared in parallel to assess the viability of each organism in the same medium for 6 h. Following the interaction between euplotes and leishmaniae, the latter were subcultured for 48 h in fresh complete medium in order to assess the reversibility of the effect on leishmaniae.

RESULTS

The dose-dependent activity of EC complex 1 against *L. major* and *L. infantum* promastigotes is shown in Fig. 2 and Table 1. Standard deviations were ≤10% of the values. At the concentration of 100 μg/ml, EC almost completely inhibited leishmania growth after just 2 h of exposure, whereas the effect of the solvent DMSO at the dilution present in EC was marginal. Concentrations as low as 20 μg/ml had good activity only after 24 h, and concentrations of 2 μg/ml were nearly ineffective (data not shown). The reduction in both the growth and survival of leishmaniae (Fig. 2; Table 1, columns A), and the irreversibility of the effect in subcultures (Table 1, columns B), strongly suggests a leishmanicidal activity. EC complex 2 was more effective against *L. major* after 48 h of treatment (Table 1); this effect was not due to the β-cyclodextrin derivative itself (DIMEB), since a blank prepared with its aqueous solution revealed no inhibitory activity against *L. major.* After incubation for 24 h, the LD_{50}s of EC complexes 1 and 2 were 4.6 ± 0.5 and 8.1 ± 1.8 μg/ml, respectively, for *L. major*, while those of both complexes for the J774 cell line were >200 μg/ml. A higher activity was noted for complex 1, paralleling the results reported in Table 1; complex 2 became more active later. Neither solubility enhancer revealed activity at the concentration present at the LD_{50} of EC. The cytotoxic activities of EC complexes 1 and 2 against the J774 cell line, as evaluated by spectrophotometric analysis and confirmed by microscopic observation, were only 30 and 20%, respectively, at the highest concentration of EC used in our experiments (200 μg/ml), and they remained at these levels after an additional 24 h of incubation. Taking into account the fact that similar activity has also been detected for the EC delivery systems (DMSO and the β-cyclodextrin derivative), the observed LD_{50} (Table 2) indicates a very low cytotoxic activity of EC against this cell line.

Complex 1, at 100 μg/ml, showed an inhibitory effect on *C. albicans*; this effect was evident when the antimicrobial activity was tested after 2 h of incubation. Subsequently, this activity was found to be partly due to DMSO (Table 2). The same is true for bacteria, in particular for strains of *Streptococcus* and *Burkholderia* spp. (Table 2). The other concentrations tested (50 and 25 μg/ml) had proportionally smaller activities (data

TABLE 2. Antimicrobial activities of the two complexes of EC and the two solvents against different microorganisms[a]

Microorganism	% of vital microorganisms after treatment with:							
	Complex 1		DMSO		Complex 2		DIMEB	
	2 h	24 h	2 h	24 h	2 h	24 h	2 h	24 h
Gram-positive bacteria								
Micrococcus luteus ATCC 9341	100	100	100	100	100	100	100	100
Staphylococcus aureus ATCC 6538	100	100	100	100	100	100	100	100
Streptococcus pyogenes ATCC 11434	40	4	85	15	100	66	100	100
Streptococcus pyogenes ATCC 8668	100	10	100	80	100	70	100	100
Gram-negative bacteria								
Escherichia coli HB101	60	48	65	90	100	80	100	92
Proteus vulgaris N9	100	100	100	100	100	100	100	100
Pseudomonas aeruginosa ATCC 15442	95	85	95	90	97	72	100	90
Burkholderia cepacia ATCC 17759	38	6	55	5	95	90	100	100
Burkholderia cepacia 1/2000	7	1	24	5	90	90	100	100
Yeast, *Candida albicans* 6	1	7	82	25	60	70	94	90

[a] EC was used at 100 μg/ml. DMSO was used as a solvent with EC complex 1, and DIMEB was used as a solvent with EC complex 2.

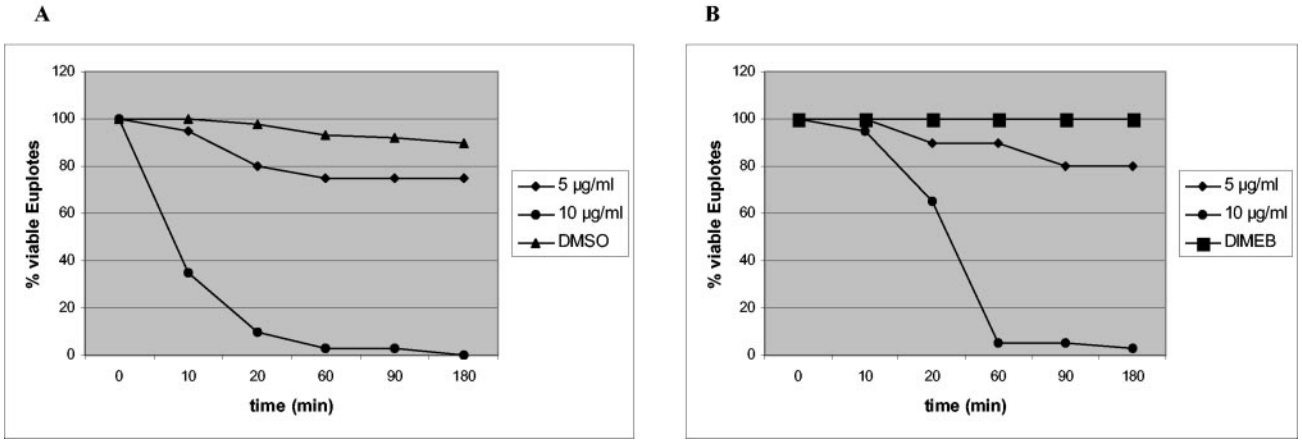

FIG. 3. Inhibitory effects of EC complex 1 and DMSO (A) and of EC complex 2 and DIMEB (B) on the growth of *E. vannus* strain TB6.

not reported). Complex 2 did not demonstrate any particular antimicrobial activity.

Evaluation of the in vitro activity of EC against the non-euplotin-producing *E. vannus* strain TB6 revealed decreases in the number of viable ciliates after 10 and 20 min of contact with EC complexes 1 and 2, respectively (Fig. 3). The activity of EC at a concentration of 10 μg/ml increased with time, reaching nearly total cytotoxicity against this protozoon after 1 h. Controls consisting of the solubility enhancers supported their lack of cytotoxic activity or its weakness: DIMEB showed no cytotoxic activity, while DMSO showed 8%, after 90 min. The interaction between *Euplotes* and leishmaniae was also assessed by using a mixture (1:4) of sterile defined seawater and physiological solution (0.9% NaCl) as the medium, permitting the viability of both protozoa for at least 6 h. When about 5×10^5 leishmaniae were placed in the medium with 2×10^4 cells of the EC producer *E. crassus* SSt22, the parasites proved to be irreversibly damaged, whereas the two individual protozoa (*E. crassus* and leishmaniae) remained viable for the duration of the experiment. The results obtained after 2 and 6 h of incubation are reported in Fig. 4A. In contrast, interaction with the non-euplotin-producing *E. vannus* strain TB6 failed to induce any detrimental effect (Fig. 4B).

DISCUSSION

This is the first report on the ability of EC, the most bioactive of the sesquiterpenes exclusively produced by strains of the marine ciliate protozoon *E. crassus*, to inhibit growth and to kill the protozoa *L. major* and *L. infantum* in vitro. A weak activity of EC complex 2 against *C. albicans* and some bacterial strains was found, while the superior activity of complex 1 is due for the most part to the solvent DMSO. Leishmanicidal activity (LD_{50}s, 4.6 and 8.1 μg/ml for complexes 1 and 2, corresponding, respectively, to 19.5 and 34.4 μM) was associated with no or moderate toxicity for murine macrophages (LD_{50}s, >200 μg/ml). EC complex 1, dissolved in DMSO, showed more-rapid activity against promastigotes, while the pronounced effect of complex 2 appeared later. Comparison of the activity of EC with that of other compounds indicates the antileishmanial potential of the natural product tested. This activity seems superior to that observed for the antiulcer agent omeprazole against different leishmanial species (27), similar to that demonstrated for some naphthoquinones and γ-pyrones (27, 28), and inferior to that determined for aphidicolin derivatives, aromatic dications, some tetra-acridine complexes, and an essential oil from the leaves of *Croton cajucara* (4, 15, 29, 43). The mechanism of killing is still a matter of debate; promastigote damage by EC was irreversible, suggesting fatal metabolic injury. Another ciliated protozoon, *Blepharisma japonicum*, exhibited an antibiotic effect against *S. aureus* linked to blepharismin, a red pigment located in the ectoplasm capable of inhibiting protein synthesis (40). Because EC is a li-

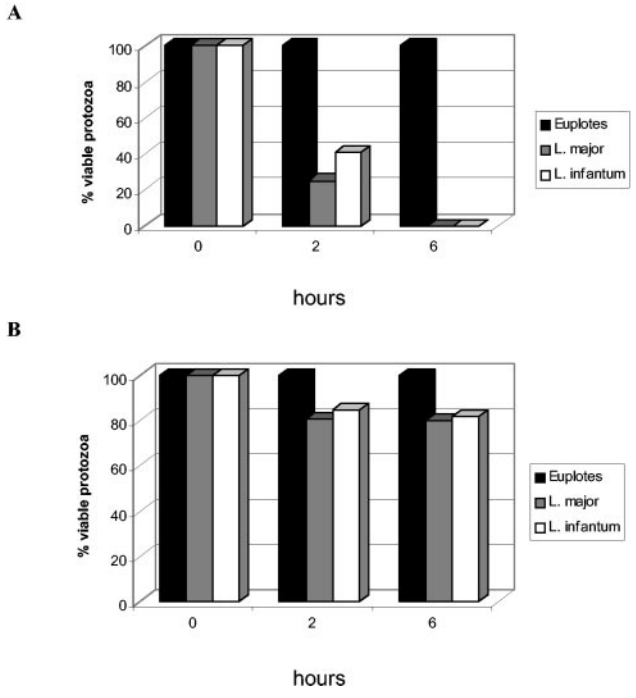

FIG. 4. Effects of the two *Euplotes* strains on the viability of *L. major* and *L. infantum*. (A) *E. crassus* strain SSt22 (euplotin-producing strain); (B) *E. vannus* strain TB6 (non-euplotin-producing strain). The *Euplotes* strains remained viable for the duration of the experiment.

pophilic compound, the activity could be targeting lipophosphoglycan, the most abundant cell surface glycoconjugate of *Leishmania* promastigotes (42, 44). The growth-inhibitory effect of EC complex 1 (and, to a lesser degree, of DMSO) on *Candida* might also be linked to an activity on the sterols present in yeast membranes.

Dini et al. (16) and Guella et al. (19) demonstrated a direct relationship between sensitivity and inability to produce euplotin in ciliate protozoa: euplotin producers, such as *E. crassus*, are resistant, while nonproducers, such as *E. vannus*, are sensitive. The results obtained by in vivo tests confirm the susceptibility of *E. vannus* to EC and describe the susceptibility of *L. major* and *L. infantum* to the natural production of EC. Considering that naturally produced terpenes and terpenoids, particularly those from plants (5, 6), demonstrated a certain antileishmanial activity and a moderate cytotoxic activity against mammalian cells, the effect observed with EC could be linked to the structure of this metabolite. Since other terpenes were obtained (20, 21, 22) from other species of *Euplotes* (*Euplotes raikovi, Euplotes rariseta*, and *Euplotes focardii*), and other compounds were obtained from *E. vannus* (23), it would be interesting to analyze the effects of these different molecules on microorganisms, and in particular on leishmaniae.

Even today, the treatment of leishmaniasis is far from satisfactory; all antileishmanial drugs are toxic. A new agent, miltefosine, registered in 2002 in India for oral treatment of visceral leishmaniasis (45), is a phospholipid analogue, developed as an anticancer drug (10). However, it shows severe toxic and teratogenic side effects (11).

In conclusion, additional experiments with in vitro and in vivo models would be useful for assessing the possible synergistic activity of this natural product with other, more toxic compounds. Moreover, Aungst and Funk (2) have described a methodology for the complete synthesis of euplotin A. Extension of this protocol to the preparation of other natural products, similar to EC from *E. crassus*, but characterized by particular selective activities, could be a future strategy.

ACKNOWLEDGMENTS

We thank L. Gradoni and M. Gramiccia (National Health Center, Rome, Italy) for the *L. infantum* strain and Enza Ferrero for careful review of the manuscript.

This work was partly supported by MIUR (ex 60% funds).

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