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Anti-*Trypanosoma cruzi* Activity of Green Tea (*Camellia sinensis*) Catechins

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The trypanocidal action of green tea catechins against two different developmental stages of *Trypanosoma cruzi* is reported for the first time. This activity was assayed with the nonproliferative bloodstream trypomastigote and with the intracellular replicative amastigote parasite forms. An ethyl acetate fraction from *Camellia sinensis* green tea leaves, which contains most of the polyphenolic compounds and the maximal trypanocidal activity, was obtained by fractionation of the aqueous extract with organic solvents. The active compounds present in this extract were further purified by LH-20 column chromatography and were identified by high-performance liquid chromatography analysis with a photo diode array detector and gas chromatography coupled to mass spectroscopy. The following flavan-3-ols derivatives, known as catechins, were identified: catechin, epicatechin, gallocatechin, epigallocatechin, catechin gallate, epicatechin gallate, gallocatechin gallate, and epigallocatechin gallate. The purified compounds lysed more than 50% of the parasites present in the blood of infected BALB/c mice at concentrations as low as 0.12 to 85 pM. The most active compounds were gallocatechin gallate and epigallocatechin gallate, with minimal bactericidal concentrations that inhibited 50% of isolates tested of 0.12 and 0.53 pM, respectively. The number of amastigotes in infected Vero cells decreased by 50% in the presence of each of these compounds at 100 nM. The effects of the catechins on the recombinant *T. cruzi* arginine kinase, a key enzyme in the energy metabolism of the parasite, were assayed. The activity of this enzyme was inhibited by about 50% by nanomolar concentrations of catechin gallate or gallocatechin gallate, whereas the other members of the group were less effective. On the basis of these results, we suggest that these compounds could be used to sterilize blood and, eventually, as therapeutic agents for Chagas' disease.

Trypanosoma cruzi is the causative agent of Chagas' disease, which is a major endemic disease in South and Central America (29). Human hosts are infected either by the triatomide insect vector bite, by blood transfusion, or by congenital transmission. The chronic phase of the disease occurs several years after infection, with cardiac and gastrointestinal pathologies being the typical clinical manifestations (6).

The main approach to the elimination of Chagas' disease in areas of endemicity is the control of transmission by the insect vector (28). On the other hand, crystal violet is the only effective chemoprotective agent for banked blood. However, the high level of toxicity of this drug has imposed severe restrictions on its use (7, 19, 21, 22).

Treatment of patients with Chagas' disease relies on two chemotherapeutic agents: benznidazole and nifurtimox. These two drugs have several limitations because they are effective but highly toxic during the acute phase of the disease and scarcely beneficial in the chronic phase (23, 25). The undesirable side effects associated with these classical trypanocidal drugs, as well as the development of resistance, are encouraging research for alternative synthetic or natural compounds effective both for the treatment of Chagas' disease and for the chemoprophylactic treatment of banked blood.

A widely used approach that has increasingly been used recently is to search for new drugs from natural sources due to its success in the detection of compounds for the treatment of some parasitic diseases (4, 6). Extracts as well as pure compounds obtained from plants have been reported to possess significant antiprotozoan activities with no side effects (2, 20).

Green tea, the dried leaf of *Camellia sinensis*, contains a variety of biologically active compounds such as polyphenols, methylxanthines, essential oils, proteins, vitamins, and amino acids (29). Most of its biological actions, such as lowering of plasma lipid levels, anti-inflammatory effects, and antimicrobial, anticancer, and antioxidant activities, are related to the polyphenol fraction, namely, tea catechins (3, 8, 11, 12, 24). These compounds, which belong to the flavan-3-ols family, have recently received considerable attention because of their potential therapeutic effects.

The aqueous extract of *C. sinensis* was found to strongly inhibit the activity of *Trypanosoma cruzi* arginine kinase (AK), a key enzyme in the energy metabolism of this parasite (C. A. Pereira et al., unpublished results). *T. cruzi* AK, which we and other investigators have cloned and characterized (1, 17, 18), is a member of the ATP-guanidinophosphotransferase family. AKs are present exclusively in invertebrates and are function-

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TABLE 1. In vitro trypanocidal activities of the different fractions obtained throughout the purification of *C. sinensis* green tea[a]

Fraction	MBC (mg/ml) for trypomastigote lysis	
	90%	50%
F1 (aqueous extract)	2	0.2
F3 (dichloromethane extract)	None	None
F4 (diethylether extract)	10	4
F5 (ethyl acetate extract)	1	0.5
F25 (LH-20-purified fraction)	0.6	0.04

[a] See Materials and Methods for details.

ally analogous to creatine kinases from vertebrates. This group of enzymes plays a fundamental role in the intracellular energy flow, acting as a reservoir for ATP through the reversible formation of a phosphagen guanidino derivative. ATP is the substrate for all members of the family (10). As the synthesis of phosphoarginine from ATP and arginine by *T. cruzi* AK is absent in human hosts, this enzyme could be an excellent target for effective chemotherapeutic agents.

In this report we describe a biologically guided screening of the effects of trypanocidal compounds extracted from *C. sinensis* on the two clinically relevant forms of *T. cruzi*: bloodstream trypomastigotes and intracellular amastigotes. We also describe the effects of these compounds on the enzymatic activity of *T. cruzi* AK.

MATERIALS AND METHODS

Reagents and plant material. Sephadex LH-20 was obtained from Amersham Pharmacia, Little Chalfont, United Kingdom. Silica gel thin-layer chromatography (TLC) plates and all organic solvents were from Merck Chemicals, Darmstadt, Germany. Other reagents were purchased from Sigma Chemical Co., St. Louis, Mo. Dried leaves of *C. sinensis*. (L.) O. Kuntze (Theaceae) were purchased from Droguería Argentina S.A., Buenos Aires, Argentina.

Mouse and parasite strains. Male specific-pathogen-free BALB/c mice from the School of Veterinary Sciences, La Plata University, Buenos Aires, Argentina, were used throughout this work. *T. cruzi* (Tulahuen strain, Tul 2 stock) bloodstream trypomastigotes were obtained from infected BALB/c mice by cardiac puncture (6).

Preparation of extracts. Dried leaves of *C. sinensis* (100 g) were powdered in a Waring blender and boiled in 1 liter of distilled water for 10 min. After the mixture was cooled, the precipitate (fraction F2) was separated from the aqueous extract (fraction F1) by filtration. Fraction F1 was extracted three times with 1 liter of each of the following organic solvents of increasing polarity: dichloromethane, diethyl ether, and ethyl acetate. Fractions corresponding to each of the organic solvent extracts were concentrated and named fractions F3, F4, and F5, respectively. The solvents were removed under vacuum in a rotating evaporator, and the dried material was resuspended in ethanol-water (1:1).

Fractionation and identification of active compounds. The ethyl acetate fraction (fraction F5) was subjected to chromatography on a Sephadex LH-20 column (24 by 2 cm), equilibrated, and eluted with absolute methanol. Sixty-two fractions of 10 ml each were analyzed by TLC on aluminum sheets (Merck silica gel 60 F_{254} plate) developed with water–1,2-dichloromethane–methanol–butanone (1:8:4:3,5) by using commercial catechins as standards and by following the general procedures described by Bohm (3). Fractions presenting lytic activity on trypomastigotes were pooled and further identified by high-performance liquid chromatography (HPLC) with a Hypersyl C_{18} (octyldecyl silane) column (125 by 4 mm) and CH_3OH–0.02 M H_3PO_4 (25:75) as the mobile phase. The conditions were as follows: elution time, 12 min; flow rate, 1 min/ml; and wavelength, 280 nm. Each absorption peak was further analyzed with a photo diode array detector (PAD). The retention times (RTs) of each of the different components in the analyzed pool were compared to those of the following standards: (±)-catechin (C; 2.136 min), (+)-epicatechin (EC; 5.223 min), (−)-gallocatechin (GC; 2.241 min), (−)-epigallocatechin (EGC; 4.863 min), (−)-catechin gallate (Cg; 10.429 min), epicatechin gallate (ECg; 7.312 min), (−)-gallocatechin gallate (GCg; 4.942 min), and (−)-epigallocatechin gallate (EGCg; 3.216 min). Data obtained by HPLC analysis were complemented by data obtained by gas chromatography coupled to mass spectroscopy (15, 32). For this purpose, samples in ethyl acetate were first derivatized with bis-trimethylsilyl trifluoracetamide and analyzed in an HP5, 30-m capillary column.

In vitro studies. The blood from *T. cruzi*-infected BALB/c mice was used to test the effects of extracts, fractions, and pure compounds by a standard World Health Organization protocol (7). Briefly, 90 μl of mouse blood containing about 5×10^4 trypomastigotes was incubated with 10 μl of each of the different extracts, fractions, or catechin dilutions in ethanol-water (1:1) in 96-well U plates at 4°C for 24 h. Control samples were run in parallel by using 10 μl of an ethanol-water (1:1) mixture. After incubation, the number of living parasites in each sample was monitored by direct microscopic examination, as described by Brenner et al. (4). Essentially, 5 μl of the blood to be tested was placed on a glass slide and covered with a coverslip (18 by 18 mm). The parasites in 50 fields were counted at a ×400 magnification. Each experiment was performed in triplicate and repeated twice. The results were expressed as the minimal bactericidal

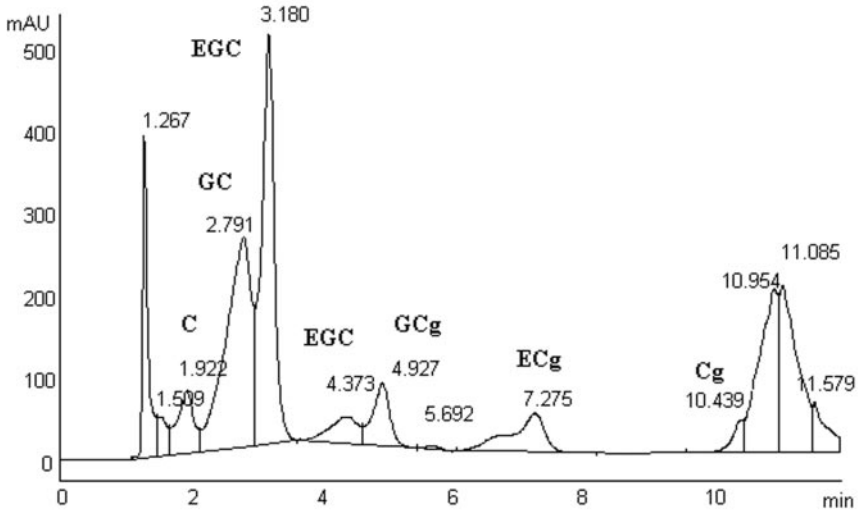
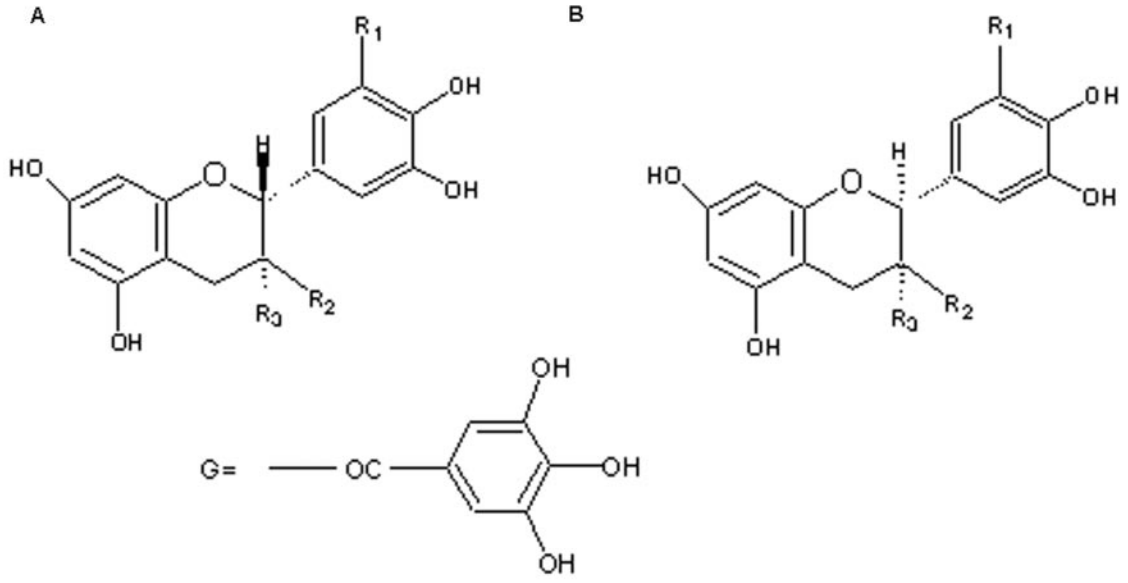


FIG. 1. HPLC-PAD chromatogram of the pool of fractions with lytic effects against the trypomastigote bloodstream form of *T. cruzi* eluted from an LH-20 column. Identifications were made according to their standard retention times, and data from analysis with a PAD confirmed the identities of the compounds. mAU, milli-absorbance units.



A	R1	R2	R3	B	R1	R2	R3
(+)-C	H	OH	H	(-)-C	H	H	OH
(+)-Cg	H	OG	H	(-)-Cg	H	H	OG
(+)-GC	OH	OH	H	(-)-GC	OH	H	OH
(+)-GCg	OG	OG	H	(-)-GCg	OH	H	OG
(-)-EC	H	H	OH	(+)-EC	H	OH	H
(-)-ECg	H	H	OG	(+)-ECg	H	OG	H
(-)-EGC	OH	H	OH	(+)-EGC	OH	H	H
(-)-EGCg	OH	H	OG	(+)-EGCg	OH	OG	H

FIG. 2. Structures of catechins.

concentration (MBC) of a fraction or compound required to kill 50% (MBC_{50}) or 90% (MBC_{90}) of the trypomastigotes present in the sample. Amastigotes were grown in Vero cells. For this purpose the cells were seeded on 12-mm-diameter glass coverslips (2×10^5 cells per coverslip) and cultured in medium 199 with Earle's salts supplemented with 1% fetal calf serum in a humidified 96% air–5% CO_2 atmosphere at 37°C (14). Each coverslip was placed in one of the wells from a 24-well plate. After 48 h of incubation, the cells on each coverslip were infected with 6×10^5 trypomastigotes derived from Vero cell cultures and cultured for another 2 h. Then, the cells were washed with phosphate-buffered saline to remove nonadherent parasites. After addition of fresh medium, the cells were incubated for 72 h, with the medium changed after 48 h. The drugs to be assayed were added either at the beginning of infection or after the cells were washed 2 h after infection. The number of infected cells and the number of intracellular parasites per cell were determined by random analysis of 500 cells per well in a Leica LB microscope, as described by Franke de Cazzulo et al. (9).

***T. cruzi* AK assay.** Homogeneous recombinant *T. cruzi* AK was expressed in *Escherichia coli* and purified as described previously (17). The native *T. cruzi* AK was purified from cytosolic extracts of epimastigote cultures. Enzyme activity was assayed as described by Pereira et al. (17). The incubation mixture contained 2 mM ATP, 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, 1 mM L-[2,3-^{3}H]arginine (0.5 μCi per assay), 25 mM HEPES buffer (pH 7.3), and an enzyme source (2 to 8 μg of protein) in a final volume of 0.2 ml. Incubations were carried out for 10 min at 30°C, and the reactions were stopped by addition of 1 ml of stop buffer (25 mM HEPES buffer [pH 7.3] containing 10 mM L-arginine and 5 mM EDTA). The mixtures were then resolved by passage through a strong anion-exchange resin (Dowex AG 1-X4, 200 to 400 mesh; Bio-Rad Laboratories, Richmond, Calif.), chloride form, mounted in 1-ml tulip columns and equilibrated with stop buffer. After the samples were loaded onto the columns, the columns were washed with 3 ml of 25 mM HEPES buffer (pH 7.3) and eluted with 2 ml of 1 M NaCl. The amount of radioactivity in the eluates was determined in UltimaGold XR liquid scintillation cocktail (Packard Instrument Co., Meriden, Conn.). The assays were carried out in triplicate.

Statistical analysis. Nonlinear regression analysis and *t* test were performed with Prism software (GraphPad Software Inc., San Diego, Calif.).

RESULTS

Biologically guided identification of trypanocidal compounds. Table 1 summarizes the results of the evaluation of the lytic activity present in each of the fractions resulting from the purification of the *C. sinensis* aqueous extract. The lytic activity of each fraction was tested with *T. cruzi* bloodstream trypomastigotes. The ethyl acetate fraction (fraction F5) had the highest trypomastigote lytic activity of all the fractions tested. Further purification of fraction F5 by Sephadex LH-20 column chromatography led to the elution of several peaks with biological activities.

Analysis by TLC showed that fraction F5 was enriched in polyphenolic compounds. The chromatographic behaviors of some of these compounds were identical to those of the commercial catechins used as reference standards, namely, EC, Cg, ECg, EGC, and EGCg.

TABLE 2. In vitro lytic effects of catechins against mouse bloodstream *T. cruzi* trypomastigotes[a]

Catechin	MBC_{50} (pM)
GCg	0.12
EGCg	0.53
GC	10.5
EGC	13
Cg	48
ECg	56
C	67
EC	85

[a] MBC_{50} were obtained by using GraphPad software. Details about the procedures are described in Material and Methods.

HPLC with subsequent analysis with a PAD (HPLC-PAD) and gas chromatography, methods currently in use for the detection of catechins in tea and organic fluids, were used to determine the compounds present in the pool of the most active fractions eluted from the Sephadex LH-20 column (3, 30). HPLC-PAD revealed the presence of C, Cg, ECg, GC, EGC, GCg, and EGCg (Fig. 1). In addition, gas chromatography of the silyl derivatives followed by mass spectrum analysis confirmed the presence of C, EC, GC, and EGC, with RTs of 24.23, 24.38, 24.60, and 24.80 min, respectively. Figure 2 illustrates the structures of the compounds identified.

The effects of these catechins on bloodstream trypomastigotes demonstrated that they have highly significant lytic activities. Comparative trypanocidal effects are shown in Table 2. The most active compounds were GCg and EGCg, with MBC_{50}s of 0.12 and 0.53 pM, respectively. Dose-response curves showed that the concentrations of most of the cathechins required to reach maximal inhibition (~90%) were about 10 to 100 nM (Fig. 3).

The same compounds were assayed for their capacities to inhibit trypomastigote infection of Vero cells and amastigote intracellular replication (Table 3). Data for the inhibition of trypomastigotes were in the nanomolar range. Increasing the concentration of catechins by 2 orders of magnitude (5 to 500 nM) caused two- and threefold increments in the lytic effects of EGCg and GCg, respectively, suggesting that the dose-response relationship must be rather flat. Neither growth inhibition nor morphological alteration of the host cells was observed.

Enzymatic assays. It has previously been found that highly diluted aqueous extracts from *C. sinensis* tea leaves significantly inhibit *T. cruzi* AK activity (Pereira et al., unpublished observations). In order to understand the biochemical interactions that could explain the trypanocidal activities of the active compounds highlighted throughout our biologically guided assay, we tested all the catechins identified for their effects on *T. cruzi* AK enzymatic activity. As shown in Table 4, the most active compounds were Cg and GCg, which were effective at nanomolar concentrations.

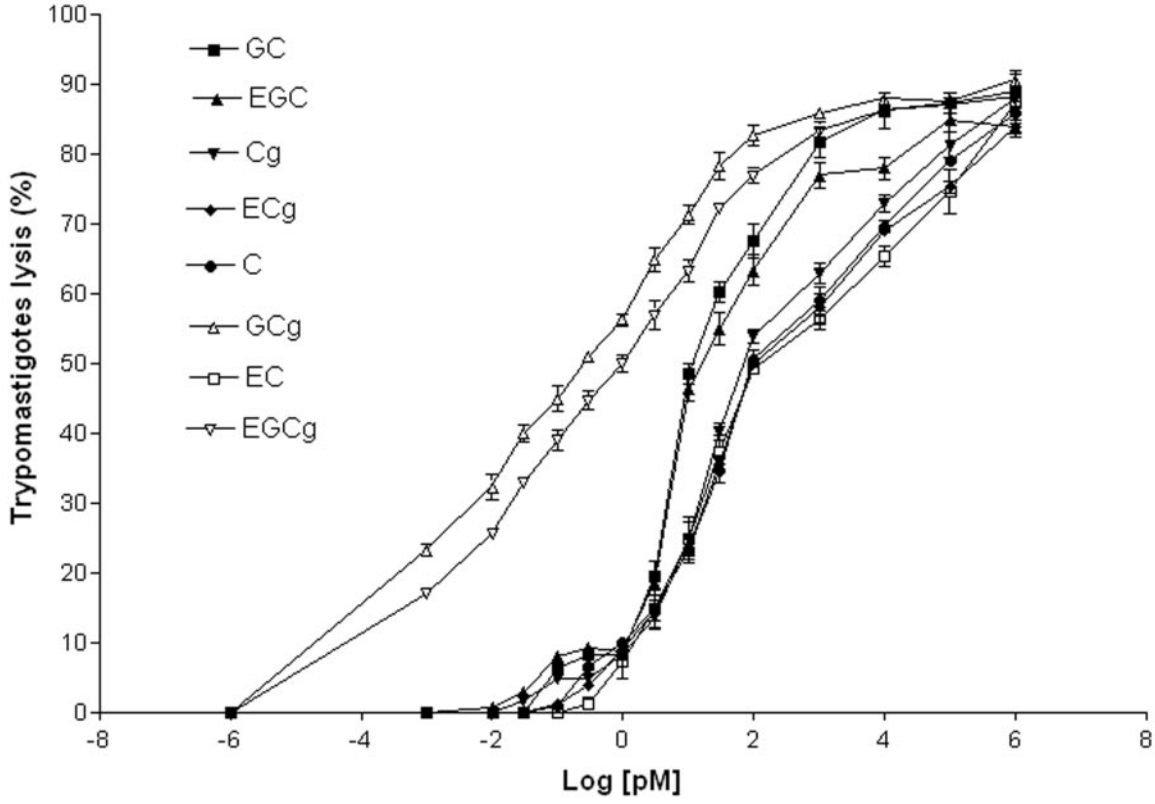


FIG. 3. Concentration dependence of the lytic effects of catechins on *T. cruzi* bloodstream trypomastigotes. The results are expressed as the percentage of lysed parasites relative to the number of parasites in the controls, to which catechins were not added. Each bars represents 1 standard deviation.

TABLE 3. In vitro lytic effects of catechins against *T. cruzi* amastigotes in Vero cell cultures

Compound (concn [nM])	% of infected cells when drug was added[a]:	
	During the infection	2 h postinfection
None	100 ± 0.0	100 ± 0.0
GCg		
5	66.00 ± 5.10[b]	68.09 ± 3.55[c]
100	45.09 ± 2.57[c]	47.05 ± 4.04[c]
500	22.12 ± 3.04[c]	25.30 ± 1.72[c]
EGCg		
5	74.50 ± 6.98[d]	73.70 ± 4.89[b]
100	52.94 ± 0.76[c]	43.13 ± 4.14[c]
500	36.42 ± 2.15[c]	34.20 ± 1.00[c]

[a] The data are expressed as the means ± standard deviations for three independent determinations.
[b] $P < 0.005$.
[c] $P < 0.0005$.
[d] $P < 0.05$.

DISCUSSION

The ethyl acetate fraction of the aqueous extract from *C. sinensis* leaves retained most of the killing activity against the protozoan parasite *T. cruzi*. The major components of this fraction belong to the flavans-3-ol family of compounds, usually known as catechins. In this report, we describe the finding of a new biological activity of catechins: a trypanocidal effect against both the infective, nonreplicative trypomastigote form and the replicative amastigote stage.

When the catechins were assayed in an infected Vero cell culture, similar results were observed when the compounds were added either at the beginning of the infection or at 2 h postinfection, when nonadherent trypomastigotes had been washed away. This suggests a direct action of some catechins against intracellular parasite forms. In agreement with a previous report (29), no visible alteration of the host cells was observed.

Both GCg and EGCg caused the lysis of approximately 50% of the bloodstream infective, nonreplicative forms of *T. cruzi* at concentrations lower than 1 pM. However, 100 nM GCg is the concentration necessary to reach the MBC_{50} for amastigotes, the intracellular, replicative form of the parasite. The different molecular structures and metabolisms of the different morphological stages of *T. cruzi*, which have been widely reported, might be the reason for the wide differences in the lytic effective concentrations of catechins found against the trypomastigote and amastigote forms.

This difference in behavior can be compared to that reported for mammary adenocarcinoma cell cultures and mammary epithelial cells. Cellular growth is inhibited approximately 50% with 5 μM EGCg in the first system, whereas 100 μM EGCg is needed to achieve the same inhibition of normal cell growth (16).

The maximal lytic activity of any of the catechins tested against trypomastigotes was never higher than 90% (Fig. 3), perhaps because nonclonal cultures were used throughout this work. Therefore, it seems that without host immunological assistance, high concentrations of catechins would be necessary to clear *T. cruzi* from the host.

The MBC_{50}s and MBC_{90}s for bloodstream *T. cruzi* forms are as low as those obtained against mammalian cell systems.

TABLE 4. Effects of catechins on *T. cruzi* AK activity

Catechin	Sp act (U/mg of protein)[a]
None	8.4 ± 1.1
C	7.8 ± 0.1
EC	6.9 ± 1.3
Cg	4.4 ± 0.6[b]
GC	6.1 ± 0.8
EGC	5.4 ± 0.7
GCg	4.5 ± 0.3[b]
EGCg	7.6 ± 0.2

[a] One unit of AK activity was defined as the amount of enzyme that produced 1 μmol of phosphoarginine per minute of incubation. The concentration of catechins in the incubation medium was 1 nM.
[b] $P < 0.05$.

Because of the key role of *T. cruzi* AK in parasite survival (1, 18) and preliminary data showing the inhibitory effect that *C. sinensis* aqueous crude extract has on this enzyme (Pereira et al., unpublished observations), we decided to test the effects of active catechins on *T. cruzi* AK enzymatic activity. We found that Cg and GCg but not EGCg inhibited approximately 50% of the enzymatic activity at concentrations of about 1 nM. The fact that of all the catechins tested only Cg and GCg strongly inhibited AK activity indicates that subtle structural differences between these compounds might enable some of them to bind specifically to critical domains on the enzyme (13, 26).

Other *T. cruzi* enzymatic targets like protein kinases were not inhibited by nanomolar concentrations of the catechins used throughout our enzymatic assays, whereas several reports indicate that micromolar concentrations are needed to inhibit mammalian enzymes, such as NADH oxidase from mammary epithelial cells (16), murine macrophage nitric oxide synthase (5), human 5α-reductase (13), and urokinase (12).

According to the results obtained from the in vitro and the enzymatic assays, we cannot conclude that parasite lysis is a consequence of *T. cruzi* AK inhibition. It has been reported that the polyphenolic groups present in catechins may be responsible for the so-called tannic action, which involves the nonspecific binding of catechins to proteins and peptides (27, 29); however, this hardly occurs at the concentrations reported here because of the trypanocidal activity and enzyme inhibition detected.

Further extensive and detailed studies of the molecular basis for the trypanocidal activities of catechins must be performed. The importance of our results is the low concentration at which the strong trypanocidal effect was observed in in vitro assays. This allows us to suggest that these compounds could be used to sterilize blood. Different assay conditions will need to be used with the infected Vero cell system in order to improve selective eradication of amastigotes without cell cytotoxicity.

Moreover, according to reported toxicological data and the bioavailability of green tea catechins (29, 33), these compounds may be active in in vivo assays with chagasic BALB/c mice, an issue that also merits further analysis.

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