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In silico and in vitro comparative activity of green tea components against *Leishmania infantum*

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ABSTRACT

Objectives: Green tea contains a predominant set of polyphenolic compounds with biological activities. The aim of this study was to investigate the antileishmanial activities of the main components of green tea, including catechin, (−)-epicatechin, epicatechin gallate (ECG) and (−)-epigallocatechin 3-*O*-gallate (EGCG), against *Leishmania infantum* promastigotes.
Methods: Green tea ligands and the control drug pentamidine were docked using AutoDock 4.3 software into the active sites of trypanothione synthetase and arginase, which were modelled using homology modelling programs. The colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure *L. infantum* promastigotes at different concentrations of green tea compounds in a concentration- and time-dependent manner. Results were expressed as 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}, respectively).
Results: In silico and in vitro assays showed that all of the green tea compounds have antileishmanial activity. EGCG and ECG were the most active compounds against *L. infantum* promastigotes, with IC_{50} values of 27.7 μM and 75 μM and IC_{90} values of 88.4 μM and 188.7 μM, respectively. Pentamidine displayed greater growth inhibition than all of the other tested compounds in a concentration- and time-dependent manner.
Conclusion: In this study, in silico and docking results were in accordance with the in vitro activity of the compounds. Moreover, EGCG and ECG showed reasonable levels of selectivity for *Leishmania*.

1. Introduction

Leishmaniasis is a neglected tropical and subtropical disease resulting in a variety of clinical manifestations in humans, ranging from severe tegumentary forms (cutaneous, mucocutaneous and diffuse cutaneous) to the fatal visceral form. This infectious disease remains a serious health problem with ever-increasing cases around the world, especially in most developing countries [1]. Leishmaniasis has been reported in 98 countries and approximately 350 million people are at risk of the infection, with 20 million people infected worldwide. The estimated incidence of the disease per annum is 2 million cases, of which 1.5 million are categorised as disfiguring cutaneous leishmaniasis and 0.5 million are the life-threatening visceral leishmaniasis [1].

Diverse clinical manifestations caused by the genus *Leishmania* are highly dependent on the host's immune condition, the parasite species as well as interactions between host immune factors and parasite components [2]. Chemotherapy using pentavalent antimonials such as Glucantime® as the first-line therapy and

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pentamidine, amphotericin B and miltefosine as second-line drugs have limitations such as toxicity, resistance, parenteral administration, variable efficacy and long administration schedules [3]. Therefore, there is an immediate requirement for new available, safe and affordable compounds for the treatment of leishmaniasis [4,5].

In silico screening, structure–activity relationship analysis, bioinformatics, and in vitro or in vivo biological evaluation are considered as valuable pharmacological tools for neglected diseases such as leishmaniasis. Knowledge of the tertiary structure of a protein used as the drug target together with computational methods can lead to the identification of potential drug binding sites. Moreover, an identified binding site can be used to characterise physicochemical properties and binding ligands on the target protein. Thus, using various methods for identification of ligand binding sites in proteins, especially recent methods for ligand binding site so-called computer-aided drug design, can contribute to finding effective and relatively safe drugs [4–7]. Target specification is one of the main steps in the process of drug development. Moreover, the biological properties of a protein depend on its physical interaction with other molecules, therefore the ability to make high-affinity and selective interactions with a drug or other biologically important molecules depends on the formation of a set of bonds and interactions. Theoretically, during specification and exploitation of a drug target, characteristic features of selected targets need to be unique to pathogens and significantly different from their host homologue or absent in the host [8,9]. Phylogenetically, trypanosomatids branch out relative to the higher eukaryotes and their cellular organisation differs from mammalian cells. Accordingly, it is possible to detect a number of targets that are specific to these pathogens [10].

Trypanothione synthetase (TS) and arginase (ARG) are unique and essential enzymes for *Leishmania* parasite survival and can serve as drug targets [11]. Synthesis of trypanothione [bis (glutathionyl)spermidine] is catalysed by TS and trypanothione reductase in the polyamine (PA) synthesis pathway and protects trypanosomatids against oxidative stress by promoting the removal of reactive oxygen species, reactive nitrogen species and other reactive species produced by the host's defence system [12]. PAs play a significant role in differentiation, proliferation and cellular redox mechanisms in *Leishmania* [13]. Thus, enzymes of the trypanothione synthesis pathway are unique and essential for trypanosomatid survival and provide promising targets for drug development.

ARG from *Leishmania* (ARG-L), located in the glycosome, is one of the major enzymes of the PA biosynthesis pathway and induces the hydrolysis of L-arginine to urea and L-ornithine, leading to PA biosynthesis. This enzyme is essential for growth, proliferation and differentiation of *Leishmania* in host cells such as macrophages and dendritic cells and is required for full infectivity of the host, and it has been exploited as a target for controlling *Leishmania* infection [14].

Food polyphenols have beneficial impacts on human health [15]. Vegetables, green tea and fruits enriched with polyphenols having antioxidant effects can prevent cardiovascular diseases [16]. Numerous natural crude and naturally derived compounds have been identified for the treatment of leishmaniasis [4]. Plant extracts and chemically defined compounds, e.g. distinct types of flavanols such as (−)-epicatechin (EC), gallocatechin 3-*O*-gallate (GCG), (−)-epigallocatechin 3-*O*-gallate (EGCG) and gallic acid have shown significant antiprotozoal effectiveness [17]. EGCG is the most abundant and valuable polyphenolic ingredient in green tea. The anti-infective activities of this flavonoid against some micro-organisms [18,19] as well as its anticancer effects, apoptosis induction and antiproliferative activity against *Trypanosoma* and *Leishmania* parasites have been studied in many investigations [20–23]. In a previous study, Feily et al. suggested that the ethanolic extract of green tea (in different concentrations) has significant leishmanicidal activity against *Leishmania major* promastigotes [24]. In another work, dos Reis et al. showed the inhibition of *Leishmania amazonensis* and rat ARGs by green tea, EGCG, catechin and EC [25].

The aim of this study was to investigate the in vitro antileishmanial activities of green tea crude extract and its main components, including catechin, EC, epicatechin gallate (ECG) and EGCG, against *Leishmania infantum* promastigotes in comparison with the control drug pentamidine. In addition, a docking simulation of the interaction between inhibitors and the structural models of ARG-L and TS was performed using AutoDock tools in order to visualise the profile of interaction with the catalytic site of the enzyme.

2. Materials and methods

2.1. Reagents

All of the tested compounds, catechin, EC, ECG, EGCG, fetal bovine serum (FBS), RPMI 1640 medium, penicillin, streptomycin, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO). All the aforementioned compounds as well as the reference drug pentamidine were refrigerated at 4 °C and were diluted in phosphate-buffered saline (PBS) (pH 7.2) at the time of incubation.

2.2. Template selection and homology modelling

The protein sequences of TS and ARG of *L. infantum* and several other kinetoplastid families were retrieved by protein Basic Local Alignment Search Tool (BLAST) in GenBank from the National Center for biotechnology information (NCBI) and Los Alamos National Laboratory websites [26]. Multiple sequence alignment was conducted using ClustalW v.1.8 to locate the regions of interest. Three-dimensional (3D) structural models of TS and ARG were created using Swiss-Model and Geno3D software and the models were validated using a set of structural validation programs, including ERRAT, WHATIF, PROCHECK and HARMONY [6].

The GenBank accession numbers of the proteins retrieved to perform either the phylogenetic analysis or the 3D modelling of ARG and TS are as follows: *Leishmania donovani* (ABG46365.1 and AEQ30090.1); *L. infantum* (AMW87957.1 and CAM69145.1); *L. major* (AMW87956.1 and CAC83968.1); *Leishmania braziliensis* (CAM43357.1 and CAM45478.1); *Trypanosoma cruzi* (EAN83844.1 and AAG15409.1); *Angomonas deanei* (EPY43326.1 and EPY38556.1); and *Bodo saltans* (CUF73863.1 and CUG92365.1).

2.3. Phylogenetic analysis

A robust phylogenetic tree was constructed using MPI-PHYLIP and MEGA. The tree was processed via the default parameters of the multiple sequence alignment programs MUSCLE and ClustalW. Curation parameters of 25, 39 and 15 were used for the minimum number of sequences for conserved position, minimum number of sequences for flanking position and maximum number of contiguous non-conserved positions, respectively. Preparation and visualisation of the constructed phylogenetic tree was conducted using maximum likelihood through a modified PHYLIP package. Philodendrons were utilised to generate the final tree diagram [6].

2.4. Virtual screening

The 3D structures of pentamidine and green tea-tested ingredients retrieved from PubChem and DrugBank databases were selected and prepared for the docking study. Virtual screening was performed using PyRx software [6,7].

2.5. Docking

The grid maps of TS and ARG-L molecules were determined with the aid of the AutoGrid part of AutoDock tools, focusing on an adequate large part of the surface to encompass the active site and also on a significant segment of the surface. AutoDock 4.3 was used to dock all of the experimental ligands and the selected green tea compounds into the active site binding pockets of the targets (TS and ARG-L). Automated docking was conducted using AutoDock 4.2 with Lamarckian genetic algorithm (LGA) to model the ligands (TS and ARG-L) interaction and binding, in which 100 multiple independent docking runs were carried out to increase the performance of docking programs. Finally, cluster analysis was performed on the observed docking values based on the root mean square (0.5 Å). Accordingly, free energy charges of binding and binding affinity were computed using AutoDock 4.2 and AutoDock Vina [6,27]. The estimated free energy charges of binding and final docking energy were calculated by the following formula:

Estimated free energy charge of binding = the sum of final intermolecular energy and torsional free energy of ligand.

2.6. Parasite culture

Promastigotes of the standard *L. infantum* strain (MCAN/IR/96/LONDON49) were obtained from Tehran University of Medical Sciences (Tehran, Iran). Promastigotes (concentration 5×10^5 cells/mL) were grown in RPMI 1640 medium supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin and 10% (v/v) heat-inactivated FBS at 24 ± 1 °C (pH 7.2). Promastigotes were then seeded in 96-well culture plates at a density of 1×10^5 cells/100 μL/well in log phase and were treated in triplicate with different concentrations of control drug and green tea compounds. The cell number was counted directly using a Neubauer chamber [28].

2.7. Measurement of cell proliferation

Plates containing promastigotes (1×10^5 cells/100 μL/well) and 10 μL of each compound mentioned above were first incubated for 24, 48 and 72 h. Subsequently, 10 μL of syringe-filtrated MTT work solution (5 mg/mL) in PBS was added to the wells and was incubated at 24 ± 1 °C for 3 h. Then, promastigote suspensions were centrifuged for 10 min and 100 μL of DMSO was added to cell sediments to dissolve formazan crystals and was incubated for 15 min. The absorbance was then read at 570 nm (reference filter 630 nm) on an enzyme-linked immunosorbent assay (ELISA) reader. Change of MTT to formazan (reduced form) is proportional to the level of energy metabolism in viable cells and a decrease in the amount of formazan indicates toxicity to the promastigotes [28,29]. Based on the optical absorbance of the treated and untreated samples and blank, the relative number of viable cells was determined by the following formula:

$$Viable\ cells(\%) = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

where *At*, *Ac* and *Ab* are the absorbance of treated, control and blank samples, respectively, represented as the mean of triplicate wells. Finally, the results were expressed as the IC_{50} (50% growth inhibition concentration).

2.8. Morphological changes of promastigotes

To evaluate changes in promastigote morphology, cells were exposed or unexposed to EGCG (30 μM), which was the most potent green tea component. Cells were then centrifuged at 1000 × *g* (2988 rpm) and PBS was used to suspend the cell pellets. Morphological changes of promastigotes were observed without staining methods using a light microscope under 100× magnification at different time points [30].

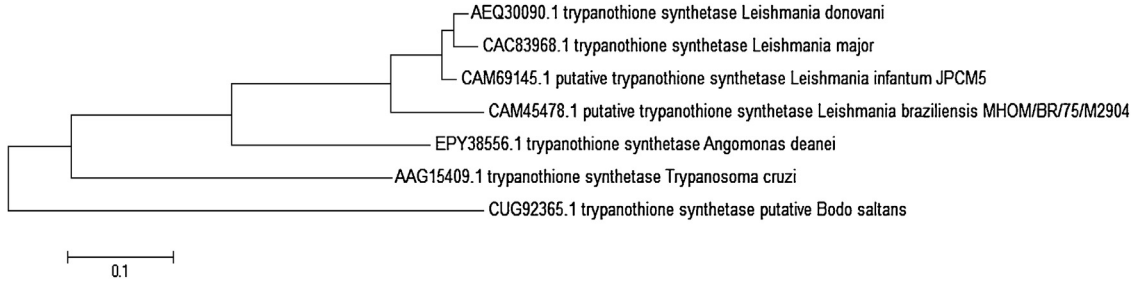


Fig. 1. Phylogenetic relationship between members of trypanothione synthetase (TS) in *Leishmania* and other kinetoplastid families.

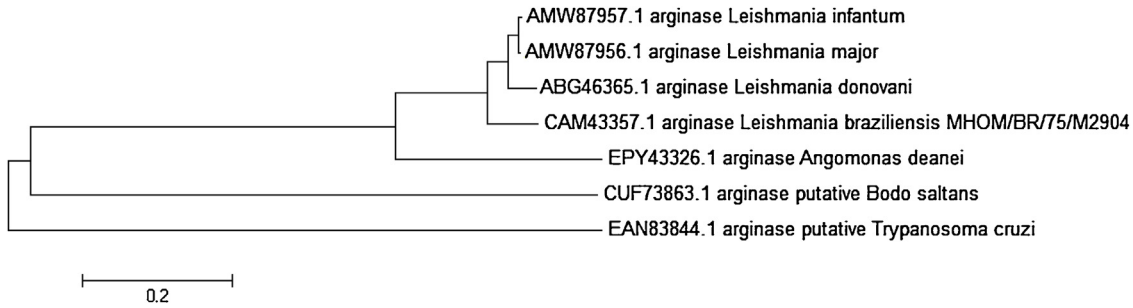


Fig. 2. Phylogenetic relationship between the members of arginase in *Leishmania* and other kinetoplastid families.

2.9. Statistical analysis

All of the results are expressed as the mean ± standard error of three independent experiments by linear regression. Data were analysed using IBM SPSS Statistics v.21.0 (IBM Corp, Armonk, NY).

3. Results

Multiple sequences of the TS and ARG-L of different kinetoplastid species were compared with each other using BLAST and ClustalW analyses. Phylogenetic trees were retrieved between the construction of TS and ARG-L, which presented a close similarity between *L. infantum*, *L. donovani* and *L. major*; however, *T. cruzi* was slightly different and formed a separate cluster distinct (Figs. 1 and 2). SWISS-MODEL and Geno3D were used to select the homologous model of the target sequence. In total, 8 and 59 templates were found for TS and ARG-L, respectively, and the best were as follows: 2vps.1.A (PDB ID) and 4ity.1.A (PDB ID) with 95.25% and 95.90% sequence identities, E values of 0.000000e-00 and 1.00000e-104 for *L. infantum* TS and ARG-L, respectively (Fig. 3). The results of validation of TS and ARG-L models and the Ramachandran plots of *L. infantum* TS and ARG-L showed 99.5% and

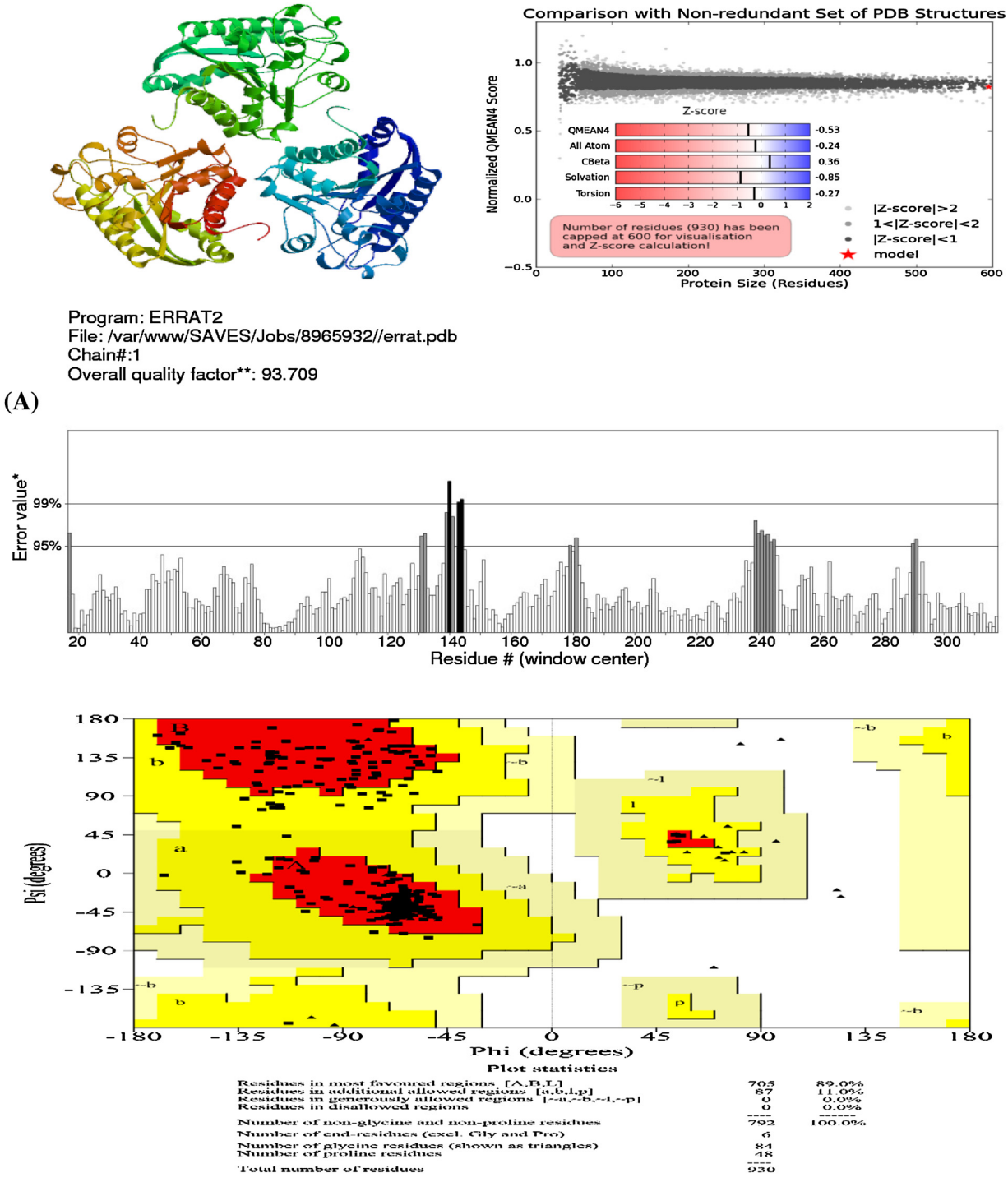
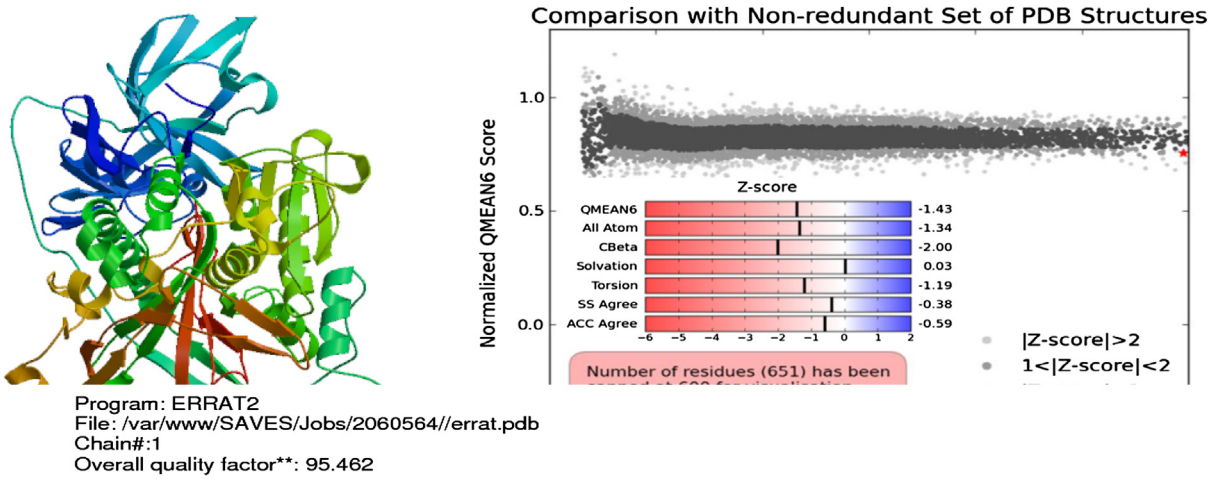


Fig. 3. (A) Trypanothione synthetase (TS) and (B) arginase (ARG-L) models from *Leishmania infantum* using homology modelling software. The detail of model validation includes PROCHECK (Ramachandran plot), and the ERRAT result has also been presented.



(B)

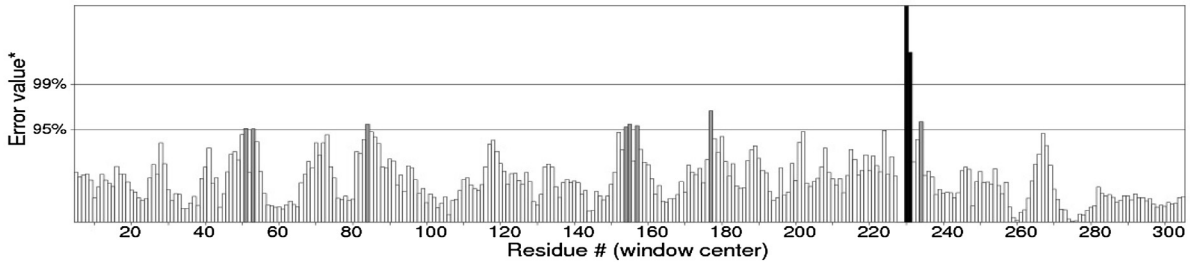


Fig. 3. (Continued)

Fig. 4. Bindings mode of (A) pentamidine to ARG-L, (B) (−)-epicatechin to ARG-L, (C) pentamidine to TS and (D) (−)-epigallocatechin 3-*O*-gallate to TS from *Leishmania infantum*. Docking results were obtained using AutoDock software, which was rendered with PyMOL. ARG-L, arginase from *Leishmania*; TS, trypanothione synthetase.

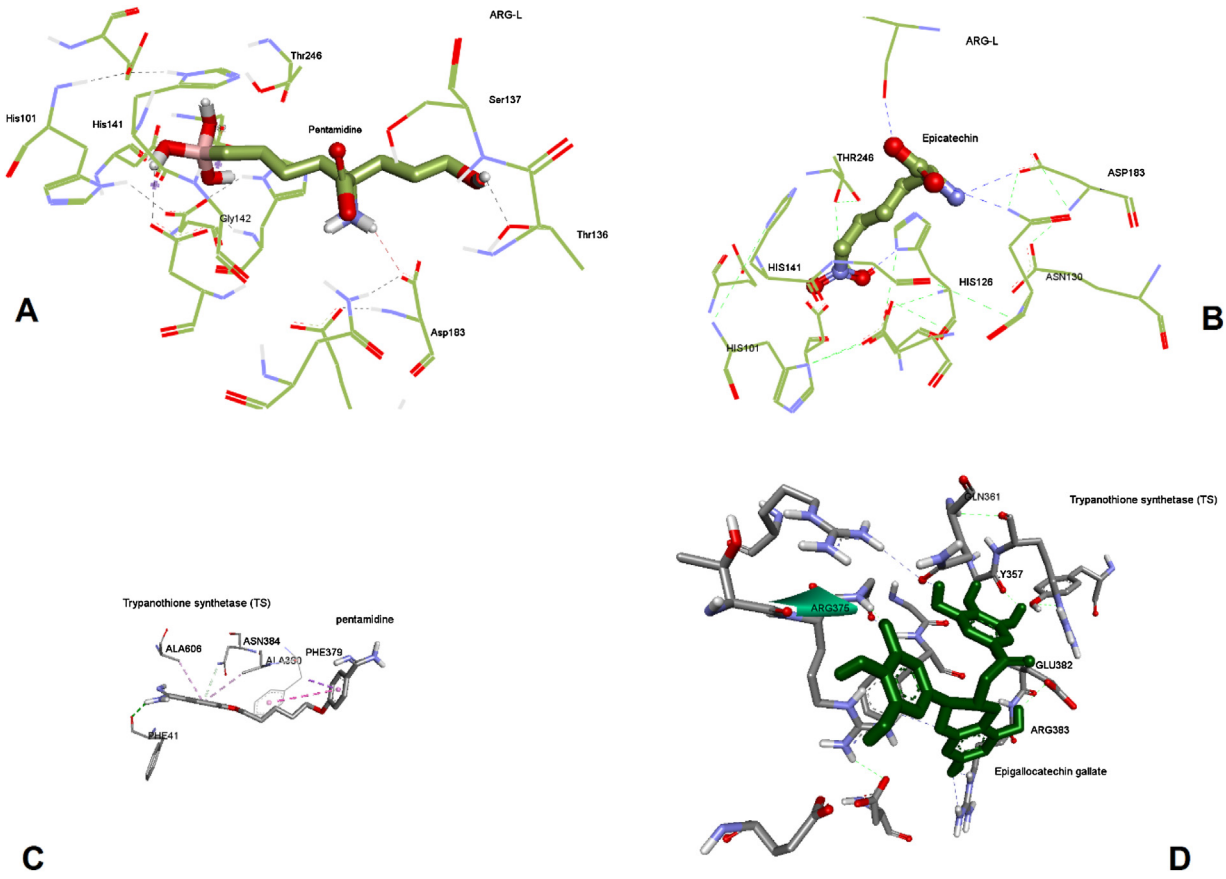


Fig. 5. (A) Pentamidine shows H-binding to the ARG-L receptor, (B) (−)-epicatechin shows H-binding to the ARG-L receptor, (C) pentamidine forms H-bonds with the trypanothione synthetase (TS) receptor and (D) (−)-epigallocatechin 3-*O*-gallate forms H-bonds with the TS receptor from *Leishmania infantum*. ARG-L, arginase from *Leishmania*; TS, trypanothione synthetase.

99% residues in acceptable (favoured and allowed) regions, respectively. The overall quality factors of the models of *L. infantum* TS and ARG-L prepared with ERRAT software were 95.4 and 93.7, respectively, indicating that the error values for residues are so small as to be negligible (Fig. 3). The binding modes of pentamidine, EC and EGCG to ARG-L or TS are depicted in Fig. 4. These compounds are likely to form H-bonds with different biding sites of amino acids both in TS and ARG-L from *L. infantum*. Pentamidine inserts in the hydrophobic pocket of ARG-L created by His101, Ser137, His141, Gly142 and Asp183 (Fig. 5A). The compound EC inserts in the hydrophobic pocket of ARG-L created by His101, His126, Asn130, His141, Asp183 and Thr246 (Fig. 5B). The compound pentamidine inserts in the hydrophobic pocket of TS created by Phe41, Phe379, Ala380, Asn384 and Ala606 (Fig. 5C). Finally, the compound EGCG inserts in the hydrophobic pocket of TS created by Gly357, Gln361, Arg375, Glu382 and Arg383 (Fig. 5D).

The estimated free energy of green tea compounds to TS and ARG-L enzymes is shown in Table 1. Of the four ligands, EGCG and EC showed the greatest binding affinity to TS and ARG-L with lesser estimated free energies (estimated ΔG) of –8.49 kcal/mol and –7.54 kcal/mol, respectively (Table 1). The in vitro leishmanicidal activities of green tea compounds and pentamidine on the growth of *L. infantum* promastigotes are shown in Table 2. The most active green tea ingredients were EGCG and ECG, with IC_{50} values of 27.71 μM and 75 μM and IC_{90} values of 88.4 μM and 188.7 μM against *L. infantum* promastigotes, respectively, after 72 h of exposure. EC, with IC_{50} and IC_{90} values of 212 μM and >512 μM, showed the lowest activity against promastigote forms of *L. infantum* after 72 h of treatment (Table 2).

Morphological changes of *L. infantum* promastigotes were observed in log-phase growth in the presence and absence of 30 μM EGCG (IC_{50}) using a light microscope at 8-h intervals.

Table 1
Estimated free energy charge of binding of green tea compounds and the control drug pentamidine to arginase (ARG-L) and trypanothione synthetase (TS) enzymes of *Leishmania infantum*.

Ligand	TS		ARG-L	
	Full fitness (kcal/mol)	Estimated ΔG (kcal/mol)	Full fitness (kcal/mol)	Estimated ΔG (kcal/mol)
Catechin	−3370.07	−7.87	−2940.01	−7.49
EC	−3433.19	−7.63	−2939.74	−7.54
ECG	−3431.1	−7.34	−2941.73	−7.40
EGCG	−3427.28	−8.49	−2939.94	−7.21
Pentamidine	−3516.03	−9.18	−3033.27	−11.35

EC, (−)-epicatechin; ECG, epicatechin gallate; EGCG, with (−)-epigallocatechin 3-*O*-gallate.

Table 2
In vitro antileishmanial activities of green tea compounds and the reference drug pentamidine against *Leishmania infantum* promastigotes after 72 h of treatment.[a]

Component	IC_{50} (μM)	IC_{90} (μM)
Catechin	94	268.7
EC	212	<512
ECG	75	188.7
EGCG	27.71	88.4
Pentamidine	10.5	34.7

$IC_{50/90}$, 50% and 90% inhibitory concentrations, respectively; EC, (−)-epicatechin; ECG, epicatechin gallate; EGCG, with (−)-epigallocatechin 3-*O*-gallate.
[a] Data are the mean ± standard error of results from triplicate experiments.

Microscopic assessment of treated promastigotes demonstrated that promastigotes began to contract and shrink 8 h after exposure to EGCG. Following 72 h of exposure, all of the treated cells showed contraction and a reduction in size compared with control promastigotes (Fig. 6).

4. Discussion

Leishmaniasis is a major health problem worldwide with no effective vaccine [1]. Treatment strategies rely exclusively on chemotherapy of affected people [31]. Inconvenience, resistance and toxicity are the main concerns with the treatment of leishmaniasis with classical drug therapies [3,32]. In the past decade, there has been an increasing trend in discovering new drug targets and vaccines against trypanosomatid parasites, focusing on their enzymes, proteins, metabolites and pathways that are unique and essential for the survival of these parasites [33,34].

TS and ARG are major *Leishmania* enzymes selected as drug targets in the present study. ARG from protozoan parasites of the genus *Leishmania* is a binuclear manganese metalloenzyme that catalyses L-arginine to ornithine in the first step of PA biosynthesis and supports *Leishmania* growth and survival in macrophages and could serve as a drug target for the treatment of leishmaniasis [14]. Trypanothione is a unique compound and is an essential player in antioxidant defence in redox mechanisms and ribonucleotide biosynthesis as well as in various specific drug resistances in parasitic trypanosomatids [12,13]. It has been suggested that certain compounds with potential anticancer effect and their derivatives might be beneficial as antiparasitic trypanosomatids agents. Therefore, in recent years, a variety of such molecules have been tested on single-celled parasites [35–37]. Plants are the main potential origin of new antiparasitic drugs. Plant extracts contain compounds assigned to diverse chemical groups, including flavonoids, alkaloids, steroids, phenylpropanoids and terpenoids [4,5].

Green tea contains a predominant set of polyphenolic compounds, especially EGCG and GCG, with antioxidative, antiangiogenic and antiproliferative effects that are potentially pertinent to different forms of chemoprevention and treatment [15]. The anticancer activity of green tea compounds on several types of cancer cell lines has previously been established and induced by cell cycle arrest and apoptosis [20,22]. Numerous studies have demonstrated that a number of trypanosomatids and unicellular eukaryotes, including *L. infantum*, possess a molecular apoptosis mechanism similar to that of programmed cell death of multicellular organisms [6,21,38]. It has also been shown that EGCG, EC and catechin have an antileishmanial influence on the growth of L. donovani amastigotes [39]. Evidence has shown the pharmacological activity of ethanolic extract of green tea against *L. major* promastigotes [24].

In the current study, the leishmanicidal activity of different concentrations of green tea compounds was assessed in a concentration- and time-dependent manner against *L. infantum* promastigotes. In silico results revealed that the flavanols such as catechin, EC, ECG and EGCG are potent inhibitors of TS and ARG-L. Docking findings were in agreement with the in vitro activity of these compounds in this experiment, and EGCG exhibited satisfactory levels of selectivity for the selected targets. Among the green tea compounds, EGCG and ECG showed maximum toxicity to the *L. infantum* promastigotes after 72 h of treatment, with IC_{50} values of 27.7 μM and 75 μM, respectively. Furthermore, EC had lesser inhibitory effect against the extracellular parasites. In a recently published article, dos Reis et al. tested EGCG, catechin and EC against ARG-L and against rat liver ARG-1 and showed that these compounds led to inhibition of ARG-L and ARG-1; however, they were more active against the parasite enzyme [25]. Moreover, enzyme kinetics demonstrated that EGCG was a mixed inhibitor of ARG-L, whereas catechin and EC were competitive inhibitors. In their study, the most potent ARG inhibitor was catechin (IC_{50} = 0.8 μM), which was followed by EC (IC_{50} = 1.8 μM), gallic acid (IC_{50} = 2.2 μM) and EGCG (IC_{50} = 3.8 μM). Docking outputs also revealed different modes of interaction of the compounds with the active sites of ARG-L and ARG-1.

5. Conclusion and future directions

The present research revealed that TS and ARG of *L. infantum* significantly differ from other trypanosomatids with the same peptides in several constructional properties, which are functionally similar at the active site of these enzymes. This study also suggested commendatory in vitro effectiveness of green tea compounds against *Leishmania* promastigotes with the help of bioinformatics analysis, which could be utilised as potential pharmacological leishmanicidal agents. It is worthwhile to perform chemical modification of the compounds and virtual screening of available compounds against the selected enzymes.

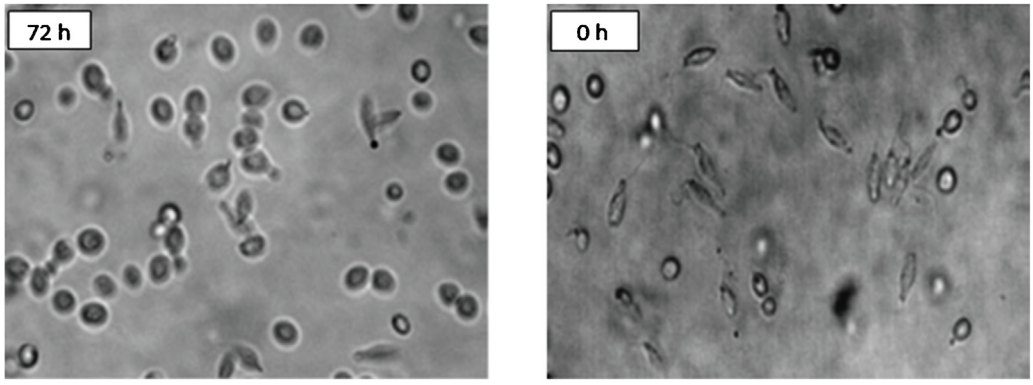


Fig. 6. Morphological changes of *Leishmania infantum* promastigotes treated with 30 μM (−)-epigallocatechin 3-*O*-gallate (EGCG) at two time points.

Moreover, assays of selected enzymes are biochemically of utmost importance. In the case of positive findings, more convincing data would arise from the experiments, showing that these compounds inhibit the active enzyme.

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Competing interests

None declared.

Ethical approval

Not required.

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