Experimental Parasitology 131 (2012) 130–132

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research Brief

Anti-*Trypanosoma cruzi* and cytotoxic activities of *Eugenia uniflora* L.

Karla K.A. Santos [a], Edinardo F.F. Matias [a], Saulo R. Tintino [a], Celestina E.S. Souza [a], Maria F.B.M. Braga [a], Gláucia M.M. Guedes [a], Miriam Rolón [b], Celeste Vega [b], Antonieta Rojas de Arias [b], José G.M. Costa [c], Irwin R.A. Menezes [d], Henrique D.M. Coutinho [a,*]

[a] *Laboratório de Microbiologia e Biologia Molecular, Universidade Regional do Cariri, Crato (CE), Brazil*
[b] *Centro para el Desarrollo de la Investigación Científica (CEDIC), Fundación Moisés Bertoni/Laboratorios Díaz Gill, Asunción, Paraguay*
[c] *Laboratório de Pesquisa em Produtos Naturais, Universidade Regional do Cariri, Crato (CE), Brazil*
[d] *Laboratório de Farmacologia e Química Medicinal, Universidade Regional do Cariri, Crato (CE), Brazil*

ARTICLE INFO

Article history:
Received 17 May 2011
Received in revised form 20 February 2012
Accepted 21 February 2012
Available online 7 March 2012

Keywords:
Chagas disease
Eugenia uniflora
Antiepimastigote activity
Cytotoxicity
Trypanosoma cruzi

ABSTRACT

Chagas disease is caused by *Trypanosoma cruzi*, being considered a public health problem. An alternative to combat this pathogen is the use of natural products isolated from fruits such as *Eugenia uniflora*, a plant used by traditional communities as food and medicine due to its antimicrobial and biological activities. Ethanolic extract from *E. uniflora* was used to evaluate *in vitro* anti-epimastigote and cytotoxic activity. This is the first record of anti-*Trypanosoma* activity of *E. uniflora*, demonstrating that a concentration presenting 50% of activity (EC_{50}) was 62.76 μg/mL. Minimum inhibitory concentration (MIC) was ⩽1024 μg/mL. Our results indicate that *E. uniflora* could be a source of plant-derived natural products with anti-epimastigote activity with low toxicity.

1. Introduction

Developing countries with traditional use of the biodiversity as medicine, including Brazil, still suffer with the so-called "neglected diseases" (Funari and Ferro, 2005), which are treated by traditional communities with plant natural products. Brazil features the largest biodiversity in the world (Elisabetsky and Costa-Campos, 1996); however only 8% have been studied in search for bioactive compounds (Garcia et al., 1996).

Chagas disease, caused by *Trypanosoma cruzi*, affects about 18 million people in the Americas (Reyes-Chilpa et al., 2008). This parasite can be transmitted to humans by triatomine insects, foods, blood and organs from infected donors, or by transplacental contamination (WHO, 2010). Currently, the chemotherapy of this disease consists mainly of nifurtimox and benzonidazole (WHO, 2010), which show a cure rate of 70–50% in the acute phase and less than 20% in the chronic phase (Dias and Dessoy, 2009). Several studies involving the analysis of natural plant products have recommended them as alternative sources of drugs against *T. cruzi*, including *Arrabidaea triplinervia* (Leite et al., 2006), *Dracocephalum kotschyi* (Saeidnia et al., 2004) and *Azorella compacta* (Araya et al., 2003).

The effects of all natural products can be limited by their toxicity. Evaluating the toxicity of active substances is one of the most important steps for the utilization of these compounds in animal models. The drugs currently utilized against Chagas disease feature high toxicity, affecting host tissues (Dias and Dessoy, 2009).

Eugenia uniflora is often used as food and medicine in folk medicine due to antimicrobial (Holetz et al., 2002) and other biological activities (Sharma et al., 2006). Known in Brazil as *pitanga*, this plant has been studied due to its antioxidant (Velazquez et al., 2003), hypotensive (Consolini and Sarubbio, 2002), photosensitizing and antibiotic modulatory (Coutinho et al., 2010a,b) activities. Several phytoconstituents of *E. uniflora* have been isolated, such as flavonoids myricitrin, quercetin and quercitrin 3-ramnoside, as well as steroids, mono- and triterpenoid compounds, tannins, anthraquinones, phenols, cineol and essential oils (Bandoni et al., 1972; Wazlawik et al., 1997).

Thus, due to the social and economic importance of Chagas disease as neglected diseases and the medicinal use of this fruit in ethnomedicine, this work evaluated the anti-*Trypanosoma* and cytotoxic activities of *E. uniflora*.

* Corresponding author. Address: Laboratório de Microbiologia e Biologia Molecular – LMBM, Departamento de Química Biológica – DQB, Universidade Regional do Cariri – URCA, Rua Cel. Antonio Luis 1161, Pimenta 63105-000, Crato (CE), Brazil. Fax: +55 (88) 31021291.
E-mail address: hdmcoutinho@gmail.com (H.D.M. Coutinho).

0014-4894
http://dx.doi.org/10.1016/j.exppara.2012.02.019

2. Materials and methods

2.1. Plant material

Leaves of *E. uniflora* were collected during the rainy season (April, 2008) in the municipality of Crato, Ceará State, Brazil. The plant material was identified by Dr. Arlene Pessoa, and a voucher specimen was deposited with identification number #3106 at the "Dárdano de Andrade Lima" Herbarium of Universidade Regional do Cariri – URCA.

2.2. Preparation of E. uniflora ethanol extract (EEEU)

A total of 200 g of leaves were dried and powdered at room temperature. The powdered material was extracted by maceration using 1 L of 95% ethanol as solvent at room temperature. The mixture was allowed to stand for 72 h at room temperature. The extract was then filtered and concentrated under vacuum in a rotary evaporator (60 °C and 760 mm/Hg of temperature and pressure) (Brasileiro et al., 2006). Each 200 g of aerial parts yield 5.6 g of extract. The EEEU was diluted using DMSO.

2.3. Cell strains

For *in vitro* studies of anti-*Trypanosoma* activity, epimastigote clone CL-B5 was used (Buckner et al., 1996). The parasites transfected with the *Escherichia coli* β-galactosidase gene (*lacZ*), were kindly provided by Dr. F. Buckner through Instituto Conmemorativo Gorgas (Panama). The epimastigotes were cultivated at 28 °C in Liver Infusion Tryptose Broth (Difco, Detroit, MI), supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA), penicillin (Ern, S.A., Barcelona, Spain) and streptomycin (Reig Jofré S.A., Barcelona, Spain), as described by Le Senne et al. (2002). Cells were harvested during the exponential growth phase. Murine J774 macrophages were used to evaluate the cytotoxic potential of the extract. This cell strain was grown in plastic 25 μL flasks with RPMI 1640 medium (Sigma) supplemented with 20% fetal bovine serum (FBS), heat inactivated (30 min, 56 °C), penicillin G (100 U/mL) and streptomycin (100 μg/mL) in a humidified, with 5% CO_2/95% air atmosphere at 37 °C. For the assay, cells in the pre-confluence phase were harvested with trypsin and kept at 37 °C in a humidified 5% CO_2 atmosphere. The cell viability measurement was a colorimetric method using resazurin as described by Rolón et al. (2006).

2.4. Reagents

Resazurin sodium salt was obtained from Sigma–Aldrich (St. Louis, MO, USA) and stored at 4 °C protected from light. The resazurin solution was prepared using 1% phosphate buffered solution (PBS), pH 7, and sterilized by filtration prior to use. Chlorophenol red-β-D-galactopyranoside (CPRG; Roche, Indianapolis, IN, USA) was dissolved in 0.9% Triton X – 100 (pH 7.4). The solutions of antibiotics penicillin G (Ern, S.A., Barcelona, Spain), streptomycin (Reig Jofré S.A., Barcelona, Spain) were prepared following the recommendations of the National Committee for Clinical Laboratory Standards – NCCLS (NCCLS, 2003).

2.5. Epimastigote susceptibility assay

The screening assay was performed in 96-well microplates with cultures that had not reached the stationary phase, as described by Vega et al. (2005). Briefly, epimastigotes were seeded at 1×10^5 mL^{-1} in 200 μL of Liver Tryptose Broth medium. The plates were incubated with the drugs in concentrations ranging between 0.1 and 50 μg/mL, at 28 °C for 72 h, at which time 50 μL of CPRG solution was added to reach a final concentration of 200 μM. The plates were incubated at 37 °C for 6 h and were evaluated using a spectrophotometer at 595 nm. Nifurtimox was used as the reference drug. Each concentration was tested in triplicate. Each experiment was performed twice separately. The efficacy of each compound was estimated by calculating the anti-epimastigote percentage (AE%) (Table 1).

Table 1
Percent parasite lysis induced by extracts of *Eugenia uniflora* against the epimastigote form of *Trypanosoma cruzi* CL-B5 strain.

Extract	Concentrations (μg/mL)	%AE	%SD	%C	EC_{50}
EEEU	100	80.83	0.1	8	
	10	64.80	3.6	0	62.76
	1	27.29	7.3	0	
Nifurtimox	10	89.1	3.3	–	
	1	54.9	0.7	–	0.91
	0.5	45.6	4.2	–	

%AE – percentual of antiepimastigote activity; %SD – standard deviation; %C – cytotoxic percentual; EC_{50} – concentration that present 50% of effect.

2.6. Cytotoxicity assays

J774 macrophages were seeded (5×10^4 cells/well) in 96-well flat-bottom microplates with 100 μL of RPMI 1640 medium. The cells were allowed to attach for 24 h in a humidified, with 5% CO_2/95% air atmosphere at 37 °C. The medium was replaced by 200 μL of medium with different concentrations of the drugs and exposed for another 24 h. Growth controls were also included. Next, 20 μL of resazurin solution with 2 mM were added and the plates were returned to the incubator for another 3 h. Resazurin reduction was determined by dual wavelength absorbance measurements at 490 and 595 nm, respectively. Each concentration was assayed three times. Medium and drug controls were used in each test. The cytotoxicity of each compound was estimated by calculating the cytotoxic percentage (C%) (Table 1).

2.7. Statistical analysis

The EC_{50} values (concentration of extract needed to necessary for produce of 50% maximal effect) were determined by linear regression analysis of the using Prism Software 5.0.

3. Results and discussion

3.1. Anti-epimastigote assay

The anti-epimastigote activity of EEEU is shown in Table 1. The results showed 80% inhibition with a concentration of 100 μg/mL, featuring EC_{50} = 62.76 μg/mL, which was quite impressive due the fact that EC_{50} lower than 500 μg/mL is considered clinically relevant (Rosas et al., 2007).

This is the first report of anti-*Trypanosoma* activity for *E. uniflora*. This activity was previously reported for the family Myrtaceae. *Siphoneugena densiflora* showed a strong effect against *T. cruzi*; however, its isolated compounds did not show similar activity (Gallo et al., 2008). Other plants of the Brazilian flora have shown substantial trypanocidal activity, such as *Ampelozizyphus amazonicus*, a plant native to the Amazon forest, containing compounds with potential for use as a prophylactic agent against that parasite (Rosas et al., 2007). The ethyl acetate fraction of the aqueous extract of *Camellia sinensis* leaves and the principal components of this fraction (catechins) demonstrated anti-trypo and amastigote

forms (Paveto et al., 2004). Trypanocidal activity has also been reported for *Dracocephalum komarovi* (Saeidnia et al., 2004), *Vitex trifolia* L. (Kiuchi et al., 2004), *A. triplinervia* (Leite et al., 2006) and *A. compacta* (Araya et al., 2003).

3.2. Cytotoxic activity

The cytotoxic activity of natural products against mammalian cells is an important point in the search for active compounds with biological activity. The results of cytotoxic activity of EEEU against J774 macrophages are presented in Table 1. A low toxicity was observed (8% to 100 μg/mL, and this toxicity was reduced to 0% with a concentration of 10 μg/mL). This low toxicity associated with trypanocidal and modulatory activity indicates that new assays need to be carried out *in vivo* to demonstrate the real potential of the EEEU against these pathogens and its effective nutraceutical potential.

The evaluation of cytotoxic activity of natural products could be demonstrated by the numerous reports using different cell models: *Calophyllum brasiliense*, tested with human lymphocytes (Reyes-Chilpa et al., 2008); *Capparis spinosa*, *Kleinia odora* and *Psiadia punctulata*, assayed with MRC-5 cells (Abdel-Sattar et al., 2010); and neolignans, such as licarin A and burchellin, evaluated against peritoneal macrophages (Cabral et al., 2010). The ethanol extract of *E. uniflora* appears to be promising in the development of more effective therapies, mainly due to the low level of toxicity *in vitro*, which allows us to proceed with *in vivo* studies for drug evaluation.

4. Conclusion

Our results indicate that *E. uniflora* (and the family Myrtaceae in general) could be a source of nutraceuticals with anti-*Trypanosoma* activity, representing an interesting alternative to combat infectious diseases as Chagas disease. This plant appears to be promising in the development of therapies, mainly due the low toxicity *in vitro*, which allows us to proceed with *in vivo* studies for drug evaluation.

Acknowledgments

The authors are grateful to the Brazilian research agencies CNPq and FUNCAP.

References

Abdel-Sattar, E., Maes, L., Salama, M.M., 2010. *In vitro* activities of plant extracts from Saudi Arabia against Malaria, Leishmaniasis, Sleeping Sickness and Chagas Disease. Phytotherapy Research 24, 1–9.

Araya, J.E., Neira, I., Silva, S., Mortara, R.A., Manque, P., 2003. Diterpenoids from *Azorella compacta* (Umbelliferae) active on *Trypanosoma cruzi*. Memórias do Instituto Oswaldo Cruz 98, 413–418.

Bandoni, A.L., Mendiondo, M.E., Rondina, R.V.D., Coussio, J.D., 1972. Survey of Argentine medicinal plants. I. Folklore and phytochemical screening. Lloydia 35, 69–80.

Brasileiro, B.G., Pizziolo, V.R., Raslan, D.S., Jamal, C.M., Silveira, D., 2006. Antimicrobial and cytotoxic activities screening of some Brazilian medicinal plants used in Governador Valadares district. Brazilian Journal of Pharmaceutical Science 42, 195–202.

Buckner, F.S., Verlinde, C.L., La Flamme, A.C., Van Voorhis, W.C., 1996. Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing beta-galactosidase. Antimicrobial Agents and Chemotherapy 40, 2592–2597.

Cabral, M.M.O., Barbosa-Filho, J.M., Maia, G.L.A., Chaves, M.C.O., Braga, M.V., 2010. Neoglicans from plants in northeastern Brazil (Lauraceae) with activity against *Trypanosoma cruzi*. Experimental Parasitology 124, 319–324.

Consolini, A.E., Sarubbio, M.G., 2002. Pharmacological effects of *Eugenia uniflora* (Myrtaceae) aqueous crude extract on rat's heart. Journal of Ethnopharmacology 81, 57–63.

Coutinho, H.D.M., Costa, J.G.M., Falcão-Silva, V.S., Siqueira-JR, J.P., Lima, E.O., 2010a. Potentiation of antibiotic activity by *Eugenia uniflora* and *Eugenia jambolanum*. Journal of Medicinal Food 13, 1024–1026.

Coutinho, H.D.M., Costa, J.G.M., Siqueira-JR, J.P., Lima, E.O., 2010b. *In vitro* screening by phototoxic properties of *Eugenia uniflora* L., *Momordica charantia* L., *Mentha arvensis* L. and *Turnera ulmifolia* L. Brazilian Journal of Bioscience 8, 299–301.

Dias, L.C., Dessoy, M.A., 2009. Chemotherapy of Chagas' disease: state of the art and perspectives for the development of new drugs. Quimica Nova 32, 2444–2457.

Elisabetsky, E., Costa-Campos, L., 1996. Medicinal plant genetic resources and international cooperation: the Brazilian perspective. Journal of Ethnopharmacology 51, 110–120.

Funari, C.S., Ferro, V.O., 2005. Uso ético da biodiversidade brasileira: necessidade e oportunidade. Revista Brasileira de Farmacognosia 15, 178–182.

Gallo, M.B.C., Marques, A.S.F., Vieira, P.C., Silva, M.F.G.D., Fernandes, J.B., 2008. Enzymatic Inhibitory activity and trypanocidal effects of extracts and compounds from *Siphoneugena densiflora* O. Berg and *Vitex polygama* Cham. Zeitschrift Naturforschung C 63, 371–382.

Garcia, E.S., Silva, A.C.P., Gilbert, B., Corrêa, C.B.V., Cavalheiro, M.V.S., Santos, R.R., 1996. Fitoterápicos. In: Farmacognosia: da planta ao medicamento. fourth ed., Campinas, Editora da UFSC.

Holetz, F.B., Pessini, G.L., Sanches, N.R., Cortez, D.A., Nakamura, C.V., 2002. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. Memórias do Instituto Oswaldo Cruz. 97, 1027–1031.

Kiuchi, F., Matsio, K., Ito, M., Qui, T.K., Honda, G., 2004. New norditerpenoids with Trypanocidal activity from *Vitex trifolia*. Chemical and Pharmaceutical Bulletin 52, 1492–1494.

Leite, J.P.V., Oliveira, A.B., Lombardi, J.A., Filho, J.D.S., Chiari, E., 2006. Trypanocidal activity of Triterpenes from *Arrabidaea triplinervia* and derivates. Biological and Pharmaceutical Bulletin 29, 2307–2309.

Le Senne, A., Muelas-Serrano, S., Fernandez-Portillo, C., Escario, J.Á., Gómez-Barrio, A., 2002. Biological characterization of a beta-galactosidase expressing clone of *Trypanosoma cruzi* CL strain. Memórias do Instituto Oswaldo Cruz. 97, 1101–1105.

NCCLS – National committee for clinical laboratory standards, 2003. Performance standards of antimicrobial disk susceptibility test. NIH, Atlanta.

Paveto, C., Güida, M.C., Esteva, M.I., Martino, V., Coussio, J., Flawiá, M.M., 2004. Anti-*Trypanosoma cruzi* activity of green tea (*Camellia sinensis*) catechins. Antimicrobial Agents and Chemotherapy 48, 69–79.

Reyes-Chilpa, R., Estrada-Muñiz, E., Veja-Avila, E., Abe, F., Kinjo, J., Hérnandez-Ortega, S., 2008. Trypanocidal constituents in plants Mammea-type coumarins. Memórias do Instituto Oswaldo Cruz 103, 431–436.

Rolón, M., Seco, E., Veja, C., Nogal, J.J., Escario, J.A., Gómez-Barrio, A., 2006. Selective activity of polyene macrolides produced by genetically modified *Streptomyces* on *Trypanosoma cruzi*. International Journal of Antimicrobial Agents 28, 104–109.

Rosas, L.V., Cordeiro, M.S.C., Campos, F.R., Nascimento, S.K.R., Januário, A.H., França, S.C., 2007. *In vitro* evaluation of the cytotoxic and trypanocidal activities of *Ampelozizyphus amazonicus* (Rhamnaceae). Brazilian Journal of Medical and Biological Research 40, 663–670.

Saeidnia, S., Gohari, A.R., Uchiyama, N., Ito, M., Honda, G., Kiuchi, F., 2004. Two new monoterpene glycosides and trypanocidal terpenoids from *Dracocephalum kotschyi*. Biological and Pharmaceutical Bulletin 52, 1249–1250.

Sharma, S.B., Nasir, A., Prabhu, K.M., Murthy, P.S., 2006. Antihyperglycemic effect of the fruit-pulp of *Eugenia jambolana* in experimental diabetes mellitus. Journal of Ethnopharmacology 104, 367–373.

Vega, C., Rolón, M., Martínez-Fernández, A.R., Escario, J.Á., Gómez-Barrio, A., 2005. A new pharmacological screening assay with *Trypanosoma cruzi* epimastigotas expressing beta-galactosidase. Parasitology Research 95, 296–298.

Velazquez, E., Tournier, H.A., Mordujovich de Buschiazzo, P., Saavedra, G., Schinella, G.R., 2003. Antioxidant activity of Paraguayan plant extracts. Fitoterapia 74, 91–97.

Wazlawik, E., Da Silva, M.A., Peters, R.R., Correia, J.F., Farias, M.R., Calixto, J.B., 1997. Analysis of the role of nitric oxide in the relaxant effect of the crude extract and fractions from *Eugenia uniflora* in the rat thoracic aorta. Journal of Pharmacy and Pharmacology 49, 433–437.

WHO – World Health Organ fact sheet No. 340, 2010. Chagas disease (American trypanosomiasis). Available in: <http://www.who.int/mediacentre/factsheets/fs340/en/> (accessed 10.09.10).