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Unlocking the *in vitro* anti-*Trypanosoma cruzi* activity of halophyte plants from the southern Portugal

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ABSTRACT

Objective: To evaluate the *in vitro* anti-*Trypanosoma cruzi* (*T. cruzi*) activity of organic extracts prepared from halophyte species collected in the southern coast of Portugal (Algarve), and chemically characterize the most active samples.

Methods: Acetone, dichloromethane and methanol extracts were prepared from 31 halophyte species and tested *in vitro* against trypomastigotes and intracellular amastigotes of the Tulahuen strain of *T. cruzi*. The most active extract was fractionated by preparative HPLC-DAD, affording 11 fractions. The most selective fraction was fully characterized by ^{1}H NMR.

Results: From 94 samples tested, one was active, namely the root dichloromethane extract of *Juncus acutus* (IC_{50} < 20 μg/mL). This extract was fractionated by HPLC, affording 11 fractions, one of them containing only a pure compound (juncunol), and tested for anti-parasitic activity. Fraction **8** (IC_{50} = 4.1 μg/mL) was the most active, and was further characterized by ^{1}H NMR. The major compounds were phenanthrenes, 9,10-dihydrophenanthrenes and benzocoumarins.

Conclusion: Our results suggest that the compounds identified in fraction **8** are likely responsible for the observed anti parasitic activity. Further research is in progress aiming to isolate and identify the specific active molecules. To the best of our knowledge, this is the first report on the *in vitro* anti *T. cruzi* activity of halophyte species.

1. Introduction

Chagas disease (CD) is a neglected tropical disease (NTD) caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), transmitted to humans and animals from the faeces of triatomine bugs (kissing bugs). It is estimated that 20–30% of humans infected with *T. cruzi* suffer with severe cardiopathy or mega-esophagus – megacolon [1]. About 8 million people are probably infected worldwide, especially in Latin America, where CD is a significant health and socioeconomic problem [2]. Moreover, CD is becoming increasing widespread in the southern area of the United States, overlapping with the poorest states [3,4]. Recently, non-vectorial *T. cruzi* infection has been increasingly recognized outside endemic areas. Europe, the United States, Canada, Australia, New Zealand and Japan host millions of at-risk Latin American immigrants [5]. Therefore, the potential of CD becoming a public health issue in that area is considerably high, mainly due to the high number of Latin American immigrants and international travellers, which may contribute for indirect transmission such as blood transfusion, organ transplantation and congenital route; and the presence of potential vectors, triatominae, in this region [5,6].

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The available drugs for CD treatment are the 5-nitrofuran, nifurtimox and the 2-nitroimidazole, benznidazole (BZ). Both drugs present low percentage cure rate, mainly in the chronic phase of the disease, when the majority of cases are diagnosed [7]. Moreover, available chemotherapeutics are highly toxic, with severe systemic side effects [7]. The problems associated with the available drugs highlight the urgent need to develop new strategies for chemotherapy against Chagas disease.

Nature has provided an important number of compounds with anti-parasitic activity. For example, quinine, first isolated from the bark of the cinchona tree (*Cinchona* sp.) was the first effective Western treatment for malaria caused by *Plasmodium falciparum*, while artemisinin from *Artemisia annua* L. is still used in malaria treatment in artemisinin-combination therapies. Noteworthy is the fact that the Nobel Prize of Physiology and Medicine 2015 was awarded to three scientists for their discoveries and development of effective drugs against parasitic infections, namely avermectin isolated from *Streptomyces avermitilis* (and its derivative ivermectin) and artemisinin from *A. annua* L. (Asteraceae) [8]. This award emphasizes that nature present unlimited chemical diversity, and highlights the value of natural products as promising alternative therapeutics towards NTDs.

Halophytes are specialized plants able to survive and thrive in saline soils. Although representing only 2% of terrestrial plant species, they are present in about half the higher plant families and have a high diversity of forms. Halophytes have evolved a complex suite of adaptations in response to the osmotic and ionic defies of saline environments that contribute to the generation of reactive oxygen species (ROS). In order to manage with excessive toxic ROS, halophytes contain antioxidant systems, including enzymes and bioactive compounds, which give them a significant plethora of other biological activities.

Although there are reports of the traditional use of different halophytic species as anti-parasitic and/or anti-helminthic agents [9], to the best of our knowledge there is no scientific information regarding the potential use of halophytes against NTDs in general, or against CD in particular. Therefore this work evaluated organic extracts made from 31 species of halophytes *in vitro* against trypomastigotes and intracellular amastigotes forms from Tulahuen strain of *T. cruzi*. The most active extract was submitted to a bio-guided fractionation, and the most promising fraction was chemically characterized by ^{1}H-NMR.

2. Material and methods

2.1. Chemicals

All chemicals used in the experiments were of analytical grade, and were purchased from VWR International (Leuven, Belgium).

2.2. Sample collection

A total of 31 indigenous (Table 1), mostly obligate, halophyte species were collected from different saline habitats of the southern Portugal (Algarve) at their full flowering time during June of 2013. The researched halophytes belong to 16 plant families and include Aizoaceae (*Mesembryanthemum crystallinum* L. and *Carpobrotus edulis* L.), Amaranthaceae (*Arthrocnemum macrostachyum* L., *Halopeplis amplexicaulis* (Vahl) Ung.-Sternb. ex Ces., Pass. & Gibelli, *Salicornia ramosissima* J. Woods, *Salicornia fragilis* P.W. Ball & Tutin, *Salsola vermiculata* L., *Sarcocornia perennis* (Mill.) A.J. Scott subsp *alpini* (Lag.) Castrov. and *S. perennis* (Mill.) subsp *perennis*), Anacardiaceae (*Pistacia lentiscus* L.), Asteraceae (*Aster tripolium* L. and *Inula crithmoides* L.), Caryophyllaceae (*Spergularia rubra* (L.) J. Presl & C. Presl), Convolvulaceae [*Calystegia* (*Convulvulus*) *soldanela* (L.) R. Br.], Cyperaceae (*Claudium mariscus* (L.) Pohl), Frankeniaceae (*Frankenia pulverulenta* L. and *Frankenia laevis* L.), Gentianaceae (*Centaurium erythraea* Rafn), Juncaceae [*Juncus acutus* (*J. acutus*) L., *Juncus inflexus* L. and *Juncus maritimus* Lam.], Lythraceae (*Lythrum salicaria* L.), Plumbaginaceae (*Limoniastrum monopetalum* (L.) Boiss., *Limonium algarvense* Erben and *Limonium lanceolatum* Hoffmanns. & Link), Polygonaceae (*Polygonum maritimum* L.) Poaceae (*Panicum repens* L., *Puccinellia maritima* (Huds.) Parl., *Spartina versicolor* Fabre), Tamaricaceae (*Tamarix africana* Poir) and Typhaceae (*Typha domingensis* Pers).

The taxonomical classification was determined by the botanist Dr Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal) and voucher specimens are kept in the herbarium of the MarBiotech laboratory (MBH01–MBH31). Different organs were collected for each species, whenever possible (Table 1). Plant material was oven dried for 3 days at 40 °C, powdered and stored at −20 °C until needed.

2.3. Preparation of the extracts

Dried samples were mixed with 80% aqueous acetone, dichloromethane and methanol (1:10, w/v) (Table 1), and extracted overnight at room temperature (RT), under stirring. Extracts were filtered (Whatman nº 4) and concentrated under reduced pressure and temperature (<40 °C). Dried extracts were dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C at the concentration of 25 mg/mL until analysis.

2.4. Evaluation of in vitro antitrypanossomal activity, cellular toxicity and selectivity

The *in vitro* antitrypanossomal activity was evaluated on L929 cells (mouse fibroblasts) infected with the Tulahuen strain of the parasite expressing the *Escherichia coli* β-galactosidase as reporter gene, according to the method described previously [10]. The extracts were tested at the concentration of 20 μg/mL, for a period of incubation of 96 h. Fractions obtained from the active extract were tested at concentrations ranging from 10 μg/mL to 100 μg/mL, also during a 96 h period. Controls with uninfected cells, untreated infected cells, infected cells treated with benznidazole at the concentration of 1 μg/mL (3.8 μM, positive control) or DMSO (1%, v/v) were used. The results were expressed as the percentage of *T. cruzi* growth inhibition