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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

Table 1

Botanical names, families, plant parts and extracts used of the 31 halophytic species included in this study.

Family	Botanical name	Plant part tested	Extracts tested
Aizoaceae	*Mesembryanthemum crystallinum* L.	Aerial organs	80% acetone, dichloromethane
	Carpobrotus edulis L.	Leaves	80% acetone, dichloromethane
Amaranthaceae	*Artrocnemum macrostachyum* L.	Aerial organs	80% acetone, dichloromethane
	Halopeplis amplexicaulis (Vahl) Ung.-Sternb. ex Ces., Pass. & Gibelli	Aerial organs	80% acetone, dichloromethane
	Salicornia ramosissima J. Woods	Aerial organs	80% acetone, dichloromethane
	Salicornia fragilis P.W.Ball & Tutin	Aerial organs	80% acetone, dichloromethane
	Salsola vermiculata L.	Aerial organs	80% acetone, dichloromethane
	Sarcocornia perennis (Mill.) A.J. Scott subsp *alpini* (Lag.) Castrov.	Aerial organs	80% acetone, dichloromethane
	Sarcocornia perennis (Mill.) A.J. Scott subsp *perennis*	Aerial organs	80% acetone, dichloromethane
Anacardiaceae	*Pistacia lentiscus* L.	Leaves	80% acetone, dichloromethane
Asteraceae	*Aster tripolium* L.	Aerial organs	80% acetone, dichloromethane
	Inula crithmoides L.	Aerial organs	80% acetone, dichloromethane
Caryophyllaceae	*Spergularia rubra* (L.) J. Presl & C. Presl	Whole plant	80% acetone, dichloromethane
Convolvulaceae	*Calystegia (Convulvulus) soldanella* (L.) R.Br.	Aerial organs	80% acetone, dichloromethane
Cyperaceae	*Cladium mariscus* (L.) Pohl	Aerial organs	80% acetone, dichloromethane
Frankeniaceae	*Frankenia pulverulenta* L.	Whole plant	80% acetone, dichloromethane
	Frankenia laevis L.	Whole plant	80% acetone, methanol, dichloromethane
Gentianaceae	*Centaurium erythraea* Rafn	Whole plant	80% acetone, dichloromethane
Juncaceae	*Juncus acutus* L.	Leaves, roots, seeds	methanol, dichloromethane
	Juncus inflexus L.	Leaves, roots	methanol, dichloromethane
	Juncus maritimus Lam.	Leaves, roots, seeds	methanol, dichloromethane
Lythraceae	*Lythrum salicaria* L.	Aerial organs	80% acetone, dichloromethane
Plumbaginaceae	*Limoniastrum monopetalum* (L.) Boiss.	Aerial organs	80% acetone, dichloromethane
	Limonium algarvense Erben	Leaves, flowers, flower stalks, roots	methanol, dichloromethane
	Limonium lanceolatum Hoffmanns. & Link	Leaves	80% acetone, dichloromethane
Polygonaceae	*Polygonum maritimum* L.	Leaves, roots	methanol, dichloromethane
Poaceae	*Panicum repens* L.	Whole plant	80% acetone, dichloromethane
	Pucinellia maritima (Huds.) Parl.	Whole plant	80% acetone, dichloromethane
	Spartina versicolor Fabre	Whole plant	80% acetone, dichloromethane
Tamaricaceae	*Tamarix africana* Poir.	Leaves	80% acetone, dichloromethane
Typhaceae	*Typha domingensis* Pers.	Aerial organs	80% acetone, dichloromethane

in extracts-tested infected cells as compared to the untreated infected cells. Active fractions were evaluated for cytotoxicity and selectivity on uninfected fibroblasts [10]. Results obtained with the fractions were expressed as IC_{50} values, calculated by linear interpolation and the selectivity index (SI) was determined based on the ratio of the IC_{50} value in the host cell divided by the IC_{50} value of the parasite.

2.5. Sample fractionation

The active dichloromethane crude extract of *J. acutus* roots was dissolved in dichloromethane at the concentration of 100 mg/mL, and fractionated by preparative HPLC-DAD (Knauer Smartline, Germany) constituted by the following modules: vacuum degasser (E4320V2), quaternary pump (EA4300V1) and the diode array detector (E4350), using a semi-preparative. Analyses were performed on a Luna 5u C18 (2) 100A, (250 × 10) mm, 5 µm particle size (Phenomenex, Spain). The mobile phase consisted on acetonitrile (solvent A) and mili-Q water (solvent B) with the following gradient: 0–40 min: 80–10% A, 40–45 min: 10–0% A, 45–55 min: 0–0% A, 55–60 min: 0–80% A, using a flow of 3.5 mL/min. The injection volume was 200 µL and the detector was set at 216 nm. 11 fractions were collected for re-testing for anti-parasitic activity.

2.6. Spectral analysis

NMR spectra were acquired on a Bruker-600 DRX (^1H NMR: 600 MHz, ^{13}C NMR: 150 MHz) spectrometer equipped with a cryo probe, in $CDCl_3$ (internal standard, for ^1H: $CHCl_3$ at d 7.26 ppm; for ^{13}C: $CDCl_3$ at d 77.0 ppm).

2.7. Statistical analyses

Results were expressed as Mean ± Standard deviation (SD), of at least three replicates. Significant differences were assessed by analysis of variance (ANOVA) or using Kruskal–Wallis test ($P < 0.05$) when parametricity of data did not prevail (SPSS statistical package for Windows, release 15.0). The IC_{50} values were calculated with GraphPad Prism V 5.0.

3. Results

From 94 samples tested, only was active, namely the dichloromethane extract of *J. acutus* with an IC_{50} value lower than 20 µg/mL. This extract was fractionated by HPLC affording 11 fractions, one of them containing a pure compound (juncunol) which were tested for cytotoxic activity against *T. cruzi* and mouse fibroblasts (L929 cells), for the determination of selectivity (Table 2). From these, fraction **8** was the most active and selective (IC_{50} = 4.1 µg/mL, SI = 1.5), and was further characterized chemically.

Although fraction **8** gave rather complex ^1H NMR spectra a qualitative analysis of the overall spectrum was possible (figure 1). The spectrum show typical signals of phenanthrenes (Phe), 9,10-dihydrophenanthrenes (dPhe) and benzocoumarins (Benz), all isolated previously from *J. acutus* (Figures 2 and 3) [11–15]. Peaks have been assigned by comparison with previously published data [10–14]. In the downfield region, between 9.1 and 8.3 ppm, the doublet signals of H-4 proton from phenanthrenes and benzocoumarins are detected. The region between 8.0 and 6.2 ppm shows all the other aromatic signals and H-12 proton from compounds with vinyl chain at C-5. Between 5.9 and 4.7 ppm H-13 protons of vinyl chain (doubledoublets) and carbinol protons of 1-hydroxyethyl (quartet) or hydroxylmethylene (singlet) are observed. Lastly, in the upfield region between 3.2 and 0.70 ppm singlet methyls and multiplets of H-9 and H-10 methylenes of 9,10-dihydrophenanthrenes are present. Trying to identify some components of the mixture, a 2D NMR experiments were performed (COSY and HMBC). The analysis of ^1H–^1H COSY evidences, in the downfield region, correlations of doublet at 9.06 d with signal at 7.08 d, doublet at 9.02 d with signal at 7.20 d, doublet at 8.99 d with signal at 7.06 d, and doublet at 8.98 d with signal at 6.48 d. The first three spin systems were assigned at phenanthrenes [12,15], while the last was attributed to a benzocoumarin [13]. The presence in the ^1H NMR of a singlet at d 3.87 (a methoxyl group), correlation observed in COSY (7.63 with 6.73 d, 6.80 with 5.54 and 5.25 d), and methyl singlets at 2.30 and 2.24 d could be attributed at 8-hydroxy-2-methoxy-1,6-dimethyl-5-vinyl-9,10-dihydrophenanthrene [12]. The presence of this metabolite was confirmed by the long-range heterocorrelations observed in the HMBC spectrum, in fact methoxyl and doublet proton (7.63 d) gave crosspeak with carbon at 156.0 ppm assigned to C-2. Furthermore, a careful analysis of COSY spectrum evidenced a proton 4.15 d (dd, J = 9.4, 7.8 Hz) correlated to 3.19 and 1.91 d, and 5.81 d with 4.98 and 4.44 (dd, J = 17.8, 1.3 Hz), these two spin system are reliable with a dimeric phenanthrenoid (Figure 3) [11]. Determinant long-range heterocorrelations of both signal at 4.44 and 4.15 ppm with carbon at 62.0 d, observed in the HMBC spectrum, supported the identification of these metabolites.

Table 2
Effect of the application of fractions from the dichloromethane extract of *Juncus acutus* (roots), on the viability of trypomastigotes and intracellular amastigotes of Tulahuen strain of *T. cruzi*.

Fraction/Compound	IC_{50} values (µg/mL)		Selectivity index
	Parasites	Mammalian cells	
1 Juncunol	67.7	63.5	0.9
2	>100.0	80.0	nd
3	34.3	40.0	1.2
4	>100.0	22.9	nd
5	>100.0	>100.0	nd
6	>100.0	12.8	nd
7	8.4	10.2	1.2
8	4.1	6.0	1.5
9	19.0	19.4	1.0
10	>100.0	80.0	nd
11	>100.0	>100.0	nd
Benznidazole[a]	1.0	625.0	625.0

[a] Positive control.

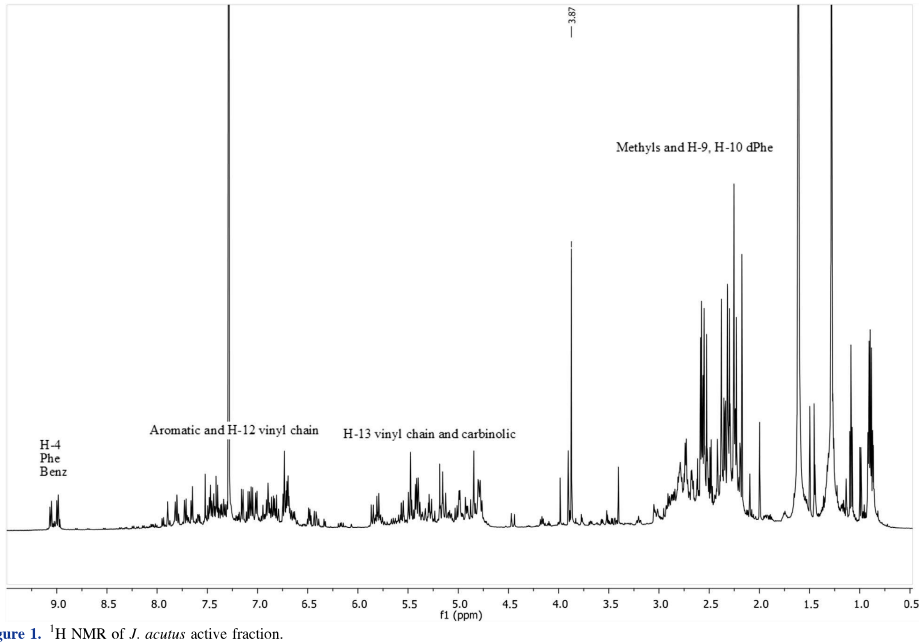

Figure 1. ^{1}H NMR of *J. acutus* active fraction.

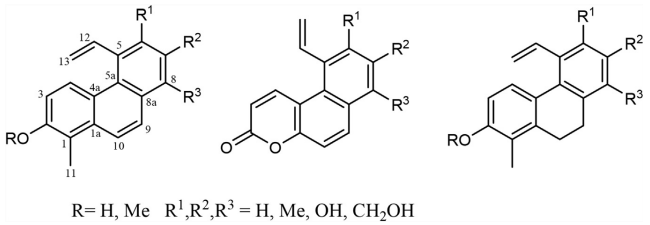

Figure 2. Structure of phenanthrene, benzocoumarin and 9,10-dihydrophenanthrene.

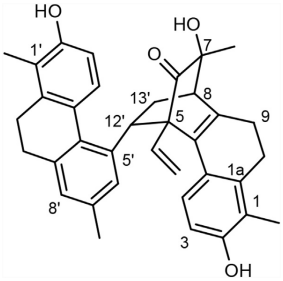

Figure 3. Structure of dimeric 9,10-dihydrophenanthrene.

4. Discussion

There are several reports on the antiparasitic activity of halophyte species. For example, seed kernels of *Caesalpinia crista* (Fabaceae) are used in traditional medicine for the treatment of malaria [16]. That activity was confirmed by *in vivo* studies using mice infected with *Plasmodium berghei* [17] and was attributed to the presence of cassane- and norcassane-type diterpenes [18]. The whole aerial organs and roots of *Inula cappa* (Asteraceae) are also traditionally used for the treatment of malaria [19,20], although the molecules responsible for that activity were not described yet. To the best of our knowledge the antiprotozoal potential of marine halophytes still remains unexplored, especially as possible candidate against CD. In this context, this work evaluated for the first time the anti-*T. cruzi* activity of organic extracts made from 31 halophyte species abundant in the southern area of Portugal.

Only one extract was able to decrease the growth of parasites, namely the dichloromethane extract of *J. acutus* roots. *Juncus* is the largest genus in the Juncaceae family comprising more than 200 species that usually grow in maritime environments. Several *Juncus* species have medicinal properties, e.g., the medulla of *Juncus effusus* is used as antipyretic and sedative in Japan and China [21]. Also, they are used in traditional medicine for the

treatment of different health problems. For example, the rhizomes of *J. acutus* are used for insomnia and the seed of *Juncus* species for the treatment of stomach disorders [21].

To assess which extract components could be responsible for the antiprotozoal activity, the active extract from *J. acutus* was fractionated by HPLC, affording 11 fractions, one of them containing a pure compound (juncunol), which were also evaluated for antitrypanossomal activity. When applied at the concentration of 67.7 μg/mL juncunol was able to reduce the parasites growth by 50%. However, it was also cytotoxic against mouse fibroblasts L929 cells. Juncunol is a dihydrophenanthrene previously isolated from of different *Juncus* species, including *J. acutus* and *Juncus roemerianus* [15,22–25], and has cytotoxic activity towards the microalga *Selenastrum capricornutum* [15], and for several mammalian tumour cell lines [22]. However, there were no reports until now on its antiparasitic activity.

Fraction **8** had the highest activity towards *T. cruzi*, and contained phenanthrenes, dihydrophenanthrenes and benzocoumarins, which were identified by ^{1}H NMR spectra analysis. Species belonging to the *Juncus* genus are one of the most prolific sources of phenanthrenes [18], which have several biological activities, including antiproliferative, antioxidant, antimicrobial and cytotoxic [22,26]. Phenanthrenoids and benzocoumarins obtained from the rhizome of *J. acutus* have *in vitro* phytotoxicity, antialgal and anti-inflammatory activities [12,14,15,27]. Recently, phenanthrene and phenanthrenoids, obtained through a bioguided fractionation of the ethanol extract of *Juncus effuses* had *in vitro* cytotoxic properties towards different cancer cell lines [28]. Also, a number of benzocoumarins are described with cytotoxic activity against different cells [29]. Moreover, some phenanthrene-derived molecules and benzocoumarins also have anti-parasitic activity. The best example is halofantrine, a phenanthrene methanol derivative used in the treatment of malaria [30]. However, this molecule is no longer recommended in current therapies due to its cardiotoxicity [30,31]. More recently, other phenanthrene-based derivatives, particularly 3-hydroxy-N′-arylidenepropanehydrazonamides, have shown potent antimalarial *in vitro* activities with high selectivity indexes [32]. Different benzocoumarin scaffolds are highly toxic towards several parasites, including the protozoans *Plasmodium* spp and *Babesia* spp [29]. Since phenanthrene-derived compounds and benzocoumarins have previously demonstrated its efficacy as antiparasitic agents, our results strongly suggest that the molecules identified in the active fraction from *J. acutus* are responsible for its antitrypanossomal activity.

To the best of our knowledge, this is the first report of the potential antitrypanosomal activity of halophyte plants in general, and of the antitrypanosomal activity of *J. acutus* extract and fractions as well as the isolated compound, juncunol. Based on our results, it is likely that the molecules identified in the active fraction from *J. acutus* are responsible for its anti *T. cruzi* activity encouraging further research. In this sense studies aiming the isolation of the bioactive (s) compound (s) of this fraction are already in progress. Moreover, structure–activity relationship (SAR) studies may also disclose a renewed interest in the pharmacological applications of these molecules, by increasing its selectivity towards parasites.

Conflict of interest statement

We declare that we have no conflict of interest.

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