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Archives of Natural and Medicinal Chemistry

Research Article

Varela J et al. Arch Nat Med Chem 2017: ANMC-105.

DOI: 10.29011/ANMC-105.000005

Identification of New Anti-*Trypanosoma Cruzi* Agents in Some Uruguayan Plants by NMR-Based Metabolomic Profiling

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Citation: Varela J, Birriel E, Nargoli J, Faral-Tello P, Robello C, et al. (2017) Identification of New Anti-*Trypanosoma Cruzi* Agents in Some Uruguayan Plants by NMR-Based Metabolomic Profiling. Arch Nat Med Chem 2017: ANMC-105. DOI: 10.29011/ANMC-105.000005

Received Date: 3 June, 2017; **Accepted Date:** 12 June, 2017; **Published Date:** 19 June, 2017

Abstract

Current available drugs to treat Chagas disease, caused by *Trypanosoma cruzi,* are ineffective, most of them are chemical synthetic drugs, and unfortunately the market of neglected diseases is not attractive for pharmaceutical industries. To overcome these problems, the developments of drugs from plants offer new possible solutions. Wild plants from Uruguay can be used as candidate drugs for the treatment of Chagas disease. Eighty ethanol extracts from several Uruguayan medicinal plants were prepared from different parts of the plants and collected from diverse conditions of soils and seasons: *Baccharis trimera, Baccharis articulata*, *Baccharis usterii*, *Hydrocotyle bonariensis, Achyrocline satureioides, Taraxacum officinalis* and *Plantago major*. As a primary screening their anti-*Trypanosoma cruzi* activity against the epimastigote form of the parasite along with the unspecific cytotoxicity in mammalian cells was evaluated. Anti-amastigote activities were determined with the selected fractions obtained from the primary screening. For the identification of the active principles from the plants, nuclear magnetic resonance based metabolomics was applied. Three *Baccharis* species, *Hydrocotyle bonariensis* and *Achyrocline satureioides* showed significant anti-proliferative activity in epimastigotes, but only the first ones were selective to the parasites.

The most active fractions of *Baccharis* species inhibited the amastigotes being selective to parasite. Through the study of the relationship between changes in chemical profiles and biological activities it was possible to identify the main active principles of the extracts as aldehyde diterpenes, and the cytotoxicity was related to furane *ent*-clerodanes. The development of drugs from wild plants with simple growing requirements, allow us to consider the future possibility of creating standardized cultivars, in order to perform *in vivo* assays and clinical trials.

Keywords: Chagas disease; anti-*Trypanosoma cruzi* agents; NMR based metabolomic; *Baccharis trimera; Baccharis articulate; Baccharis usteri.*

Abbreviations:

B. articulate	:	*Baccharis articulate*
B.trimera	:	*Baccharis trimera*

B.Usteri :	*Baccharis usteri*
CPRG :	Chlorophenol red-β-D-galactopyranoside
2D NMR:	Two dimension nuclear magnetic resonance
DMSO :	Dimethylsulfoxide
FID :	Free Induction Decay
HBMC :	Heteronuclear Multiple Bond Correlation
HSQC :	Heteronuclear Single Quantum Coherence
MTT :	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
NMR :	Nuclear Magnetic Resonance
PLS-DA:	Partial Least Square-Discriminant Analysis
SI :	Selectivity Index
TLC :	Thin Layer Chromatography
TMS :	Tetramethylsilane
T. cruzi :	*Tryponosoma cruzi*

Introduction

Chagas disease is caused by the flagellate protozoan *Trypanosoma cruzi* (*T. cruzi*). It is transmitted to humans through bites and concomitant defecation of different triatomine species, all of which potentially carry the parasite in their contaminated feces. Other modes of transmission include blood transfusion or blood infection from infected mother to her child, or by oral ingestion of parasite contaminated food [1]. The disease is a serious endemic illness that affects millions of people generating health, economic and social problems in the countries affected [2]. It is widespread in Central and South America, affecting 21 countries in these regions. It has been estimated that this disease affects between 5.7 million people, while 60 million remain at risk. Population mobility around the world and residence in endemic areas potentiate the possibility of expansion of the disease. A Brazilian doctor, Carlos Ribeiro Justiniano Chagas, discovered the disease in 1909, however there are still no effective chemotherapies for all its clinical forms [3]. Like other neglected diseases it is a major health problem that continues to grow as a result of inadequate therapy and the lack of an effective vaccine [4]. People suffering from neglected tropical diseases constitute an unattractive market to private sector research and for investment development [5]. Unfortunately, the current available drugs for the treatment were found to have many kinds of toxicity [6]. A fundamental problem regarding neglected tropical diseases is the lack of engagement of pharmaceutical companies in the research and development of new, not expensive and effective treatments, required for the population affected [7]. Therefore, research to uncover new solutions to treat these diseases with more effective and safer medications is of utmost importance [8]. Natural products continue to be an important source of chemotherapeutic agents, particularly those used to treat infectious diseases. Out of the 162 new chemical entities approved as anti-infective drugs by regulatory agencies over the period 1981-2002, ninety nine (61%) were from a natural origin [9]. Natural products have a huge chemical diversity in which it is possible to find new agents for treatment of neglected tropical diseases. In this study, wild Uruguayan plants with known antibacterial and antifungal activity [10,11] were tested eighty-nine against *T. cruzi,* supported by previous work of our group where plant extracts with antifungal and antibacterial properties presented significant *in vitro* and *in vivo* anti-*T. cruzi* activity [12]. *Baccharis, trimera, Baccharis articulata*, *Baccharis usterii*, *Hydrocotyle bonariensis, Achyrocline satureioides, Taraxacum officinalis* and *Plantago* major were the species selected to study as anti- *T. cruzi* agents. These Uruguayan plants are able to grow in different types of soil, sandy or rocky [13]. On the other hand, the biological activity of plants might vary according to the harvest season because of secondary metabolism modifications, which result in different chemical compounds and in variable quantities.

To study the changes in the plant metabolic profiles we applied Nuclear Magnetic Resonance (NMR) spectroscopy based metabolomic. By means of NMR-based metabolomic we search for the profiling of the active principles. Nuclear magnetic resonance is traditionally considered as a prime tool in profiling, characterization, and structure elucidation of molecules, and is becoming increasingly popular for metabolomic studies [14]. Apart from its low sensitivity, NMR technique has some favorable features that give it an edge over other analytical techniques such as being non-destructive, requiring a simple sample preparation, shorter analysis time, easer quantification, and non-selectiveness towards specific metabolites. NMR has been used in the metabolic profiling of various types of samples in combination with different multivariate data analysis tools [16]. This combination has been very useful in distinguishing closely related plant species [17], as well as to identify bioactive compounds [18].

The aim of this work was to identify the potential anti-*T. cruzi* activity in plants with antifungal and antibacterial properties. We studied the relationship between the metabolic variation of plants in the different environmental conditions (type of soil and collection season) and the anti-*T. cruzi* activity in order to profile the active principles by NMR-based metabolomics. We also evaluated the chemical profile variation in correlation with the cytotoxic activity in order to profile the cytotoxic entities.

Results

Plants collection and extraction

Plant samples employed in this study were collected from different geographical locations of Uruguay (Figure. S1, Supplementary material) according to the following criteria of selection:

i) Weather season: the collection was done during the four seasons present in Uruguay (summer: December to March, autumn: March to June, winter: June to September, and spring: September to December).

ii) Type of soil: sandy or rocky soils.

iii) Botanic section: the whole plant, roots, leaves and flowers (in the cases of flowering time) were collected.

To obtain most of the metabolites and study the complete chemical profile, ethanol extracts were prepared from different parts of the plants, aerial parts like leaves and flowers, or roots with yields between 4 to 20 % (Table 1).

Plant	Plant section	Yielda (%)
Baccharis trimera	Leaves and flowers	15-18
Baccharis articulata	Leaves and flowers	13-16
Baccharis usterii	Leaves and flowers	13-17
Hydrocotyle bonariensis	Leaves	13-20
Hydrocotyle bonariensis	Stems and rhizomes	6-11
Hydrocotyle bonariensis	Flowers	4-7
Achyrocline satureioides	Leaves and flowers	8-12
Plantago major	Leaves and flowers	12-16
Plantago major	Roots	5-9
Taraxacum officinallis	Leaves and flowers	10-15

[a] The yields were informed as a range between three extraction preparations

Table 1: Yields of ethanol extracts obtained with the selected species.

Biology

The anti-proliferative capacity of eighty ethanol extracts was evaluated against the epimastigote form of *T. cruzi*, Tulahuen 2 strain. The unspecific cytotoxic activity of the extracts was assessed in a model of mammalian cells, J-774.1 murine macrophage-like cells. The selectivity to parasite is 5 expressed as the selectivity index (SI) calculated as the ratio between the IC_{50} against the mammalian cells and the IC_{50} against the parasite (Tables 2) and (Table 3). A selectivity index higher than 1.0 indicates that the extract is more toxic to the parasites than to the mammalian cells. For the ethanol extracts of the species, *Taraxacum officinalis* and *Plantago major*, no anti-epimastigote activity was observed (IC_{50} > 100 µg/mL, Table S1, supplementary material) and the ethanol extract of *Achyrocline satureioides* although with certain anti-*T. cruzi* activity, presented high cytotoxicity to mammalian cells. Consequently, they were not included in further studies.

Plant Species	Season (date) and soil type	Location	Plant section	Anti *T.cruzi* act. IC_{50}[a] (µg/mL)	cytotoxicity IC_{50}a (µg/mL)	SI[b]
Achyrocline satureioides	Summer (Feb 2012),rocky	Villa Serrana	Leaves and flowers	45.0 ± 5.1	48.0 ± 2.3	1.1
	Summer (Feb 2012),sandy	Jauregui berry	Leaves and flowers	64.7 ± 3.6	72.0 ± 2.4	1.1
	Summer (Feb 2012), sandy	Santa Teresa	Leaves	23.3 ± 0.4	11.0 ± 1.3	0.5
	Summer (Feb 2012), sandy		Stems and rhizomes	7.6 ± 0.9	12.0 ± 1.2	1.6
	Summer (Feb 2012), sandy		Flowers	5.2 ± 1.6	7.8 ± 1.2	1.5
	Summer (Feb2014), sandy		Stems and rhizomes	61.5 ± 3.1	<7.8	<0.1
	Summer (Feb 2014), sandy		Flowers	22.4 ± 3.2	<7.8	<0.3
	Autumn (Jun 2012),rocky/sandy	Cuchilla Alta	Leaves	46.5 ± 5.0	37.0 ± 1.3	0.7

Hydrocoty leb-onariensis	Autumn (Jun 2012), lake	Facultad de Ciencias	Stems and rhi-zomes	55.0 ± 4.2	15.6 ± 1.5	0.3
	Winter (Aug 2013), lake		Stems and rhi-zomes	24.1 ± 3.2	7.8 ± 0.9	0.3
	Spring (Set 2012), sandy		Stems and rhi-zomes	56.5 ± 3.8	15.0 ± 1.3	0.3
	Summer (Mar2013), sandy	Barra del Chuy	Stems and rhi-zomes	44.4 ± 2.9	<7.8	<0.2
	Summer (Feb 2013), sandy		Leaves	89.6 ± 3.3	13.0 ± 1.1	0.1
	Summer (Feb 2013), sandy		Stems and rhi-zomes	36.7 ± 2.9	14.0 ± 1.3	0.4
	Summer (Feb 2013), sandy	Punta del Este	Flowers	8.6 ± 0.9	7.8 ± 0.8	0.9
	Winter (Aug 2013), sandy		Stems and rhi-zomes	22.3 ± 2.9	<7.8	<0.3

[a] IC_{50} is the concentration that cause the 50% inhibition of growth; values are given in µg/mL and are the mean ± S.D. [b]SI: Selectivity index = IC_{50} cytotoxicity / IC_{50} *T. cruzi*. Reference drug: Nifurtimox, IC_{50}, *T. cruzi*= 7.0 ± 1.0, IC50, J774-1= 316.0 ± 0.5 values are given in µM and are the mean ± S.D. SI= 45.

Table 2: Anti-epimastigote and cytotoxic activities of the ethanol extracts of *Achyrocline satureioides* and *Hydrocotyle bonariensis.*

Plant species	Season (date) and soil type	Location	Plant section	anti-T. cruzi act. IC50a(µg/m)	Cytotoxicity IC50a(µg/m)	SI[b]
Baccharis trimera	Summer (Feb 2012), rocky	**Villa Serrana**	Leaves and flow-ers	51.5 ± 7.6	67.0 ± 2.1	1.3
	Autumn (Jun 2012), rocky		Leaves and few flowers	49.6 ± 2.9	37.0 ± 1.4	0.7
	Spring (Dec 2012), rocky		Leaves	52.0 ± 2.7	37.0 ± 1.3	0.7
	Winter (Aug 2013), rocky		Leaves and few flowers	86.5 ± 4.9	40.0 ± 1.4	0.5
	Summer (Feb 2014), rocky		Leaves and flow-ers	64.7 ± 2.1	> 150	>2.3
	Autumn (Jun 2012), rocky/ sandy	**Cuchilla Alta**	Leaves and few flowers	43.4 ± 2.9	73.0 ± 2.4	1.7
	Summer (Feb 2012), sandy		Leaves and flow-ers	97.5 ± 3.5	38.0 ± 1.6	0.4
	Summer (Feb 2013), sandy	**Punta del Este**	Leaves and flow-ers	68.1 ± 2.0	75.0 ± 2.6	1.1
	Winter (Aug 2013), sandy		Leaves and flow-ers	18.3 ± 2.4	75.0 ± 2.5	4.1
	Summer (Feb 2012), sandy	**Santa Teresa**	Leaves and flow-ers	68.9 ± 11.7	37.5 ± 1.3	0.5
	Spring (Oct 2013), sandy	**Cabo Polonio**	Leaves	30.9 ± 2.5	> 150	>4.8
	Summer (Feb 2013), rocky	**Pozos Azules**	Leaves and flow-ers	13.6 ± 1.2	37.0 ± 1.3	2.7
	Summer (Mar 2013), sandy	**Barra del Chuy**	Leaves and flow-ers	43.4 ± 2.7	75.0 ± 2.2	1.7
	Spring (Dec 2012), rocky		Leaves	55.2 ± 3.9	53.0 ± 2.2	0.9

Baccharis usterii	Winter (Aug 2013), rocky	**Villa Serrana**	Leaves and flowers	87.5 ± 6.2	50.0 ± 2.4	0.6
	Summer (Feb 2014), rocky		Leaves	78.4 ± 3.6	65.0 ± 2.7	0.8
	Summer (Feb 2013), rocky	**Pozos Azules**	Leaves	21.9 ± 1.7	60.0 ± 1.8	2.7
	Summer (Feb 2012), rocky		Leaves	23.0 ± 3.0	94.0 ± 2.6	4.1
Baccharis articulata	Spring (Dec2012),rocky	**Villa Serrana**	Leaves	99.5 ± 2.3	35.0 ± 1.5	0.4
	Winter (Aug 2013), rocky		Leaves and flowers	51.9 ± 4.2	60.0 ± 2.1	1.2
	Winter (Aug 2013), sandy		Leaves	16.6 ± 2.0	100.0 ± 2.9	6
	Spring (Dec 2013), sandy		Leaves and flowers	33.7 ± 2.4	60.0 ± 1.9	1.8
	Summer (Feb 2014), rocky	**Punta del Este**	Leaves and flowers	59.8 ± 2.7	150.0 ± 2.7	2.5
	Summer (Feb 2013), Sandy	**Santa Teresa**	Leaves	43.6 ± 3.2	30.0 ± 2.4	0.7
	Summer (Feb 2012), sandy	**Jauregui berry**	Leaves	22.5 ± 4.0	78.0 ± 1.7	3.5

[a] IC_{50} is the concentration that cause the 50% inhibition of growth; values are given in µg/mL and are the mean ± S.D. [b]SI: Selectivity index = IC_{50} cytotoxicity / IC_{50} *T. cruzi*. Reference drug: Nifurtimox, IC_{50}, T. cruzi= 7.0 ± 1.0, IC50, J774-1= 316.0 ± 0.5 values are given in µM and are the mean ± S.D. SI= 45.

Table 3: Anti-epimastigote and cytotoxic activities of the ethanol extracts of *Baccharis* spp.

For some of the most active Baccharis spp. ethanolic extracts the anti-amastigote activities were also determined (Table 4).

Plant species	Season (date),Soil type	Location	Plant section	anti-*T. cruzi* act.IC_{50} [a] (µg/mL	SI[b]
Baccharistrimera	Winter (Aug 2013),sandy	**Punta del Este**	Leaves and flowers	18.1 ± 1.1	4.1
	Spring (Oct 2013),sandy	**Cabo Polonio**	Leaves	27.9 ± 2.5	>5.4
	Summer (Feb2013), rocky	**Pozos Azules**	Leaves and flowers	9.9 ± 0.8	3.7
Baccharisarticulata	Summer (Feb2012), rocky		Leaves	39.0 ± 5.3	2.4
	Summer (Feb2014), rocky	**Villa Serrana**	Leaves	22.3 ± 1.1	4.5
Baccharisusterii	Summer (Feb2013), rocky	**Pozos Azules**	Leaves	28.1 ± 6.9	2.1
Reference drug	Benznidazole			0 .9 0 ± 0 . 0 5 [c]	-

[a] IC_{50} is the concentration that cause the 50% inhibition of growth; values are given in µg/mL and are the mean ± S.D. b SI: Selectivity index = IC_{50} cytotoxicity (Table 3) / IC_{50} *T. cruzi* (amastigotes).c IC_{50} is the concentration that cause the 50% inhibition of growth; values are given in µM and are the mean ± S.D.

Table 4: Anti-amastigote and cytotoxic activities of the ethanol extracts of *Baccharis* spp.

Metabolomic studies

The chemical profiles of the different ethanolic extracts were determined with the ^{1}H NMR spectrum of each sample. As expected, the samples with different biological activities showed significant variations on their chemical profiles. The relationship between chemical profile variations with biological activity was evaluated by multivariate data analysis. The chemical profile is the ^{1}H NMR spectrum of ethanolic extract of each sample.

Multivariate data analysis is an essential step of any metabolomics study. These methods are used to reduce the dimensionality of multivariate dataset and thus enable to recognize possible differences or similarities among the samples. In order to identify the metabolites responsible for *in vitro* inhibition of *T. cruzi* growth, a supervised method, i.e. PLS-DA, was used. In this method the actual data from the bioactivity assay against epimastigotes was used as a *y*-variable. The PLS-DA analysis, 5-components explained 78.9% of the variance and Q2 value of 0.29, was found effective in separating the high and low active samples of *Baccharis* species (see example for *Baccharis articulata* in Figure 1A. In order to identify the metabolites responsible for the cytotoxic activity of the extracts PLS-DA method was used in which the actual data from the cytotoxicity assayed against J-774.1 murine macrophage-like cells was used as the *y*-variable. A good separation was obtained between different cytotoxic activity groups, with a 2-components model that explained 55.8% of the variance and Q2 value of 0.21 (see example for *Baccharis articulata* in Figure 1B.

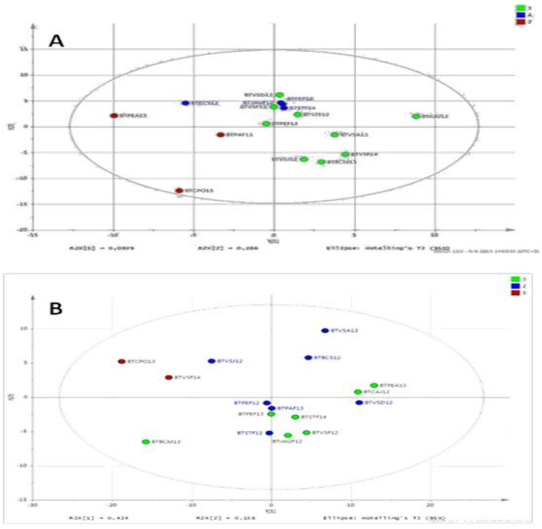

Figure:1A) Score plot of PLS-DA results obtained from all ^{1}H NMR data of *Baccharis articulata* samples and its *anti-Trypanosoma cruzi* activity showing PC1 and PC2. Blue: not interesting activity. Red: Moderate biological activity. Green: Relevant biological activity.

Figure:1B) Score plot of PLS-DA results obtained from all 1H NMR data of *Baccharis articulata* samples and its cytotoxic activity showing PC1 and PC2. Blue: Relevant toxicity. Red: Low toxicity. Green: Some toxicity. Yellow: No toxicity.

The PLS-DA analysis, 8-components explained 97.4 % of the variance and Q2 value of 0.18, for *Baccharis trimera* samples using the biological activity against epimastigotes as the *y*-variable, showed that the most active group was separated from the other samples. Groups with low biological activity were not well separated by this method (Figure 2A).

To identify the metabolites responsible for the cytotoxic activity, the PLS-DA method was applied in which the actual data from the cytotoxicity assay against J-774.1 murine macrophage-like cells was used as the *y*-variable. This PLS-DA method, 9-components explained 99.2 % of the variance and Q2 value of 0.18, showed no separation between the groups of samples with different activities (Figure 2B).

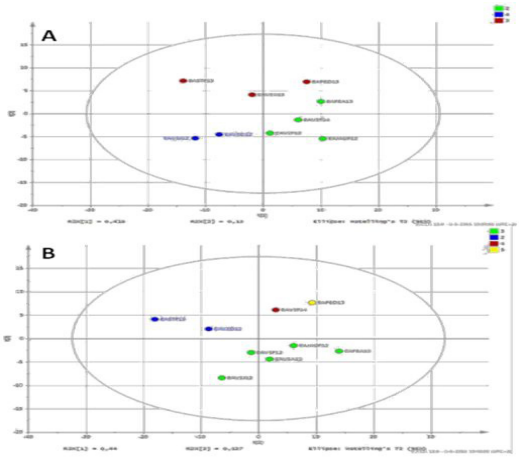

Figure 2:A) Score plot of PLS-DA results obtained from all ^{1}H NMR data of *Baccharis trimera* samples and its anti-*Trypanosoma cruzi* activity showing PC1 and PC2. Blue: not interesting activity. Green: Moderate biological activity. Red: Relevant biological activity.

Figure 2:B) Score plot of PLS-DA results obtained from all $_1$H NMR data of *Baccharis trimera* samples and its cytotoxic activity showing PC1 and PC2. Blue: Relevant toxicity. Red: No toxicity. Green: Some toxicity.

For *Baccharis usterii* we performed a PLS-DA using the biological activity against epimastigotes of the samples as *y*-variable in order to identify the active principles. It was not possible to perform PLS-DA using cytotoxicity against J-774.1 murine macrophage-like cells like *y*-variable for two reasons.

1. Not enough number of samples required for a prediction.
2. The few samples evaluated in this study, presented the same cytotoxic activity. However, observing the biological activity changes and the constant cytotoxicity, it was possible to infer that the compounds which presented anti-*T. cruzi* activity and un-specific cytotoxicity were different.

Discussion

We developed a ranking to classify extracts by their ability to inhibit the growth of *T. cruzi*: when the $IC_{50} \leq 10\mu g/mL$ the

extract displayed "strong biological activity", when the $10 < IC_{50} \leq 40$ the extract displayed "relevant biological activity", when the $40 < IC_{50} \leq 100$ the extract displayed "moderate biological activity", and when the $IC_{50} > 100$ µg/mL the extract was "not considered" for further studies. This classification was based in previously described natural extracts with anti-parasitic activity [19]. Some *Achyrocline satureioides* extracts from samples collected in Villa Serrana and Jaureguiberry locations during summer of 2012 showed moderate biological activity (Table 2). The extracts of *Hydrocotyle bonariensis* showed strong to moderate anti-

T. cruzi activity. Particularly, the flowers of *Hydrocotyle bonariensis*, collected in summer of 2012, were the most active followed by the stems and rhizomes, collected in the same season, lacking of activity in the leaves (Table 2).

It was observed relevant antiproliferative activity of *Baccharis* genus plants (Table 3). *Baccharis trimera* samples showed moderate biological activity, having also in some cases relevant activity, i.e. leaves and flowers collected in Pozos Azules and Punta del Este during summer and winter of 2013 from rocky and sandy soils, respectively, and leaves collected in Cabo Polonio during spring of 2013 from sandy soil. The same was found in *Baccharis articulata* showing moderate biological activity and cases of relevant activity, i.e. leaves collected in Villa Serrana and Jaureguiberry during summers of 2014 and 2012 from rocky and sandy soils, respectively, and leaves and flowers collected in Punta del Este during winter of 2013 from sandy soil. *Baccharis usterii* samples also showed moderate biological activity with cases of relevant activity, i.e. leaves collected in Pozos Azules during summer of 2013 from a rocky soil.

The cytotoxic activity of the extracts from *A. satureioides*, *H. bonariensis*, *B. trimera, B. articulata*, and *B. usterii* was assessed in a model of mammalian cells, J-774.1 murine macrophage-like cells. To define the selectivity to the parasite the selectivity index (SI) was calculated as the ratio between the IC_{50} against the mammal cells and the IC_{50} against the parasite. The SI for *A. satureioides* and most of the extracts from *H. bonariensis* were less or near to 1.0 transforming these extracts into not adequate for further studies (Table 2). The *B. trimera* extracts presented modest cytotoxicity in several cases.

The best selectivity rates were obtained in the cases of leaves collected in Cabo Polonio during spring of 2013 from a sandy soil, and leaves and flowers collected in Punta del Este, Pozos Azules, and Villa Serrana during winter and summer of 2013 and summer of 2014 from sandy and rocky soils, respectively. *B. usterii* only showed good SI in leaves collected in Pozos Azules during summer of 2013 from a rocky soil. *B. articulata* showed, like its congener *B. trimera,* low cytotoxicity, also resulting in good SI. The best results were observed in the leaves collected in Villa Serrana and Jaureguiberry during summer of 2014 and 2012 from rocky and sandy soils, respectively. In general, the results obtained showed that the presence of flowers in plants appear to be an important factor in the decreasing of the selectivity index (Table 3).

For some of the most relevant extracts, leaves and flowers of *B. trimera* from Punta del Este sandy soils (winter of 2013), leaves of *B. trimera* from Cabo Polonio sandy soils (spring of 2013), leaves and flowers of *B. trimera* from Pozos Azules rocky soils (summer of 2013), leaves of *B. articulate* from Villa Serrana rocky soils (summers of 2012 and 2014), and leaves of *B. usterii* from Pozos Azules rocky soils (summer of 214 2013), the anti-amastigote activities were determined (Table 4). The amastigotes are the relevant pathogenic form living intracellularly in the host in nests; consequently moderate activity of one extract reaffirms the relevance of this agent as a potential candidate for therapy. The results against amastigotes were in agreement with the findings against epimastigotes. The best selectivity indexes were from the two extracts of *B. trimera*, from Cabo Polonio and Pozos Azules, rocky and sandy soils respectively (Table 4). The best performance against the amastigotes was displayed by the extract from rocky soils ($IC_{50} = 9.9 \pm 0.8$ µg/mL) and the best selectivity was evidenced in the extract from Sandy soils (SI > 5.4). The other extracts showed moderate to strong selectivity indexes (higher than 2.1).

Among the metabolites described in *B. trimera* are saponins, clerodane diterpenes and flavonoids such as quercetin, gencavanin, cirsimaritin, hispidulin, apigenin, luteolin, nepetin, genkwanin and rutin [20,21]. In *B. articulata* were described flavonoids, tannins and terpenes, being terpenoids the major compounds, including several triterpenes [22]. Two neoclerodan diterpenes, articulin acetate and articulin, have also been described [23]. Other components are crisosaponic acid, resinic acid, oleanolic acid, lupeol and the flavonoids such as santonin, absintin, luteolin, quercetin, genkwanin, acacetin, 7, 4-dimethyl-apigenin, cirsimaritin, salvigenin, jaceidin, jaceosidin and chondrillasterol. In the flowers it was found a furan diterpene, barticulidiol, and a clerodane diterpene, bachotricuneatin [24]. For *B. usterii* 4, 5-*O*- dicaffeoylquinic acid and 7-hydroxy-5,4'-dimethoxy-flavone have been described [25].

On the other hand, our experience in finding natural compounds as new agents against *T. cruzi* indicates that terpenoid compounds are responsible for the biological activity. In particular diterpenes and triterpenes [12,26]. But we could not rule out the synergistic action between various components of the extracts. In the PLS-DA score plot of *B. articulata* samples using the biological activity as *y*-variable (Figure 1A), three groups were clearly separated and directly related with their biological activity. The analysis showed that an aldehyde, related with diterpene ^{1}H NMR signals (δ= 9.72, 9.56, 9.36, 5.44-5.28, 5.16, 5.12, 2.80 and 2.08 ppm, Figure 3), was the most related compound with the differences in the biological activity in the samples [21,27,28]. 2D NMR studies allow us to infer the structure of the active principle from *B.articulata* as 7-hydroxy-ent-cleroda-3-en-16-lactone-18-al (Figure 3).

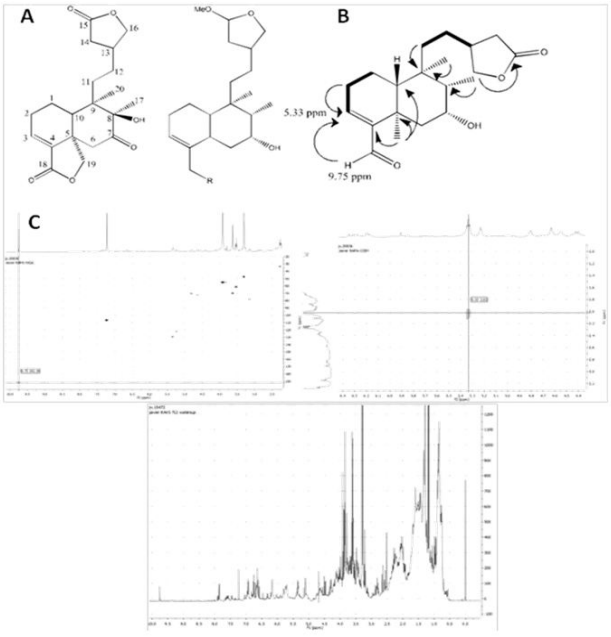

Figure: 3A) Diterpenes reported by Dai et al. (*from Baccharis articulata*).

Figure: 3B) Proposed structure of active principle from the identified signals in the *Baccharis articulata* 2D NMR related with diterpenes already reported in Baccharis species by Dai et al.

Figure: 3C) HMBC and COSY observed correlations. Arrows denote the key HMBC correlations and bold lines indicate ^{1}H-^{1}H correlations.

The analysis of the PLS-DA score plot of *B. articulata* samples using the cytotoxic activity as y-variable (Figure 1B) suggested that the metabolites responsible for the anti-*T. cruzi* and cytotoxicity in *B. articulate* extracts were not the same. The relevant ^{1}H RMN signals (δ=6.64, 5.08, 3.80, 3.08, 2.44-2.32, 1.52 and 1.12-1.08 ppm, Figure 3) for this analysis, cytotoxic effects, were correlated with *ent*-clerodane furane diterpenes reported for *Baccharis* species [20,29]. (Figure 4).

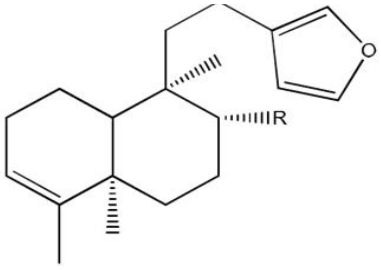

Figure 4: Furane diterpenes reported by Zdero et al. in Baccharis species.

For *B. trimera* samples the analysis of the PLS-DA using the biological activity against epimastigotes as *y*-variable (Figure 2) showed that the main ^{1}H NMR signals (d= 9.60, 9.40, 6.36, 5.68, 5.24, 5.20, 2.28, 0.80 and 0.68 ppm) correlated with the most active group of samples, and were similar to the ones found in *B. articulata.* It was also possible to observe signals corresponding to aldehyde-diterpenes related with compounds like those shown in (Figure 3), but we cannot affirm that was the same compound proposed for *B. articulata*.

In the analysis of the PLS-DA using cytotoxicity assay against J-774.1 murine macrophage-like cells as a *y*-variable (Figure 2B) it was possible to identify the signals (δ= 9.44, 9.28, 6.36 and 5.72 ppm) that 9 correlated with group 5 (no toxicity) and these signals were the same that those found for the aldehyde diterpenes. For *B. usterii* the PLS-DA model considering the biological activity against epimastigotes of the samples as *y*-variables was used to identify the active principles. Considering the information of the loading plot for the data of the most active sample (with the following ^{1}H NMR signals: 9.32-9.12 ppm), we suggested that the compounds with variability correlated to biological activity were also the aldehyde- diterpenes. The three *Baccharis* species were compared by thin layer chromatography (TLC) using Brady reagent (2,4-dinitrophenylhydrazine) to detect aldehydes, vainillin-sulfuric reagent to detect terpenes, and UV light (254 nm) to detect UV-chromophores (Figure 5). It was observed that the three species showed compounds with aldehyde groups in their structures (positive with Brady reagent) and also these compounds were terpenes (positive with vainillin-sulfuric reagent). It was noteworthy that for *B. trimera,* the presence of at least two aldehyde diterpenes in the extract was observed. We compared the ^{1}H NMR of the three *Baccharis* species under study to show the similarities on the principal components of the samples (Figure 6). In the ^{1}H NMR spectrum the principal metabolites observed were diterpenes.

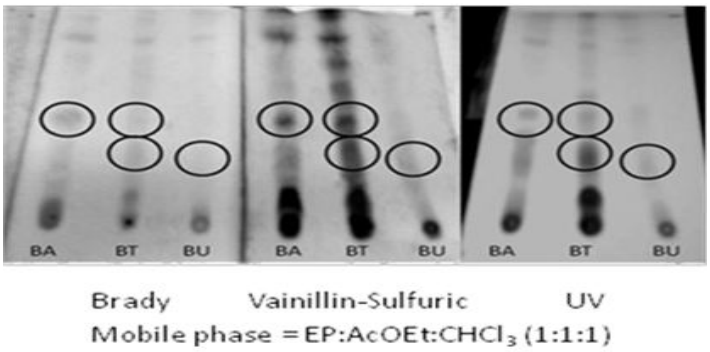

Figure 5: TLCs of *Baccharis species* (BA - *Baccharis articulata*, BT - *Baccharis trimera*, BU - *Baccharis usterii*). The circles show the positive revealed zones.

Materials and methods

Plant material

The selected plants were collected considering the following variables. For seasonal variation the samples were collected through four seasons present in Uruguay (summer: December to

March, autumn: March to June, winter: June to September, and spring: September to December). For soil variation characteristics of land, sandy and rocky soils were considered. For all the plants employed in this study, the whole plant, roots, leaves and flowers (in the cases of flowering time) were collected.

Botanical identification

Botanical identification of the species collected was performed by Prof. Eduardo Alonso Paz (Laboratorio de Botánica, Facultad de Química-Universidad de la República, Uruguay). One specimen of each of the collected samples is preserved in the herbarium of our laboratory and in the herbarium of Facultad de Química-Universidad de la República, Montevideo, Uruguay. The registration number are: *Baccharis trimera*-Number 4402, *Baccharis articulate* - Number 4403, *Baccharis Usterii*-Number 4404, *Hydrocotyle bonariensis* -Number 4406, *Taraxacum officinale*-Number 4407,*Plantago major* -Number 4408, *Achyrocline Satureioides*-Number 4409.

Extracts preparation

The plant material was washed with distilled water and then oven dried at 40°C to constant weight. Ten grams of dried plant material was weighed and extracted with 150 mL of EtOH (95 %) for 48 h at room temperature and protected from light. After 48 h it was filtered and the same extraction procedure was repeated. The solvent was evaporated under reduced pressure at no more than 40°C, to yield the ethanol extract.

Anti-*Trypanosoma cruzi* activity

Anti-epimastigotes activity

Trypanosoma cruzi epimastigotes (Tulahuen 2 strain) were grown at 2°C in an axenic milieu (BHI-Tryptose) supplemented with 5 % fetal bovine serum. Cells from a 10-day-old culture (stationary phase) were inoculated into 50 mL of fresh culture milieu to generate an initial concentration of 1 ´ 10^6 cells/mL. Cell growth was followed by measuring the absorbance of the culture at 600 nm every day. Before inoculation, the media was supplemented with the indicated quantity of the extracts or isolated compounds from a stock solution in DMSO, in which the final concentration never exceeded 0.4 %. The control was run in the presence of 0.4 % DMSO and in the absence of studied extracts. No effect on epimastigotes growth was observed due to the presence of up to 1 % DMSO in the culture media. The percentage of inhibition (PGI) was calculated as follows: PGI (%) = {1- [(Ap - A0p)/(Ac - A0c)]} ´ 100, where Ap = A_{600} of the culture containing the drug at day 5; A0p = A_{600} of the culture containing the extract just after addition of the inocula (day 0); Ac = A_{600} of the culture in the absence of extract (control) at day 5; A0c = A_{600} in the absence of the drug at day 0. In order to determine IC_{50} values, 50% inhibitory concentrations, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding extract. At day 5, the absorbance of the culture was measured and related to the control. The IC_{50} value was taken as the concentration of extract needed to reduce the absorbance ratio to 50 % and the average of three different experiments [12]. Nifurtimox, the current available used drug, was used as the positive control drug.

Anti-amastigote activity

Vero cells were plated and infected with β-galactosidase expressing trypomastigotes of the Dm28c strain [30] at a ratio of 10 parasites per cell. After 2 h of infection, non-internalized parasites were washed out and different concentrations of compounds ranging from 0.375 µM to 25 µM were added. After 72 h of amastigote replication, monolayers were washed and assays were developed using CPRG as substrate as previously described [31]. Color changes were quantified by measuring the absorbance at 570 nm using an ELx800 Universal Microplate Reader (BioTek Instruments Inc., Winooski, VT). Wells with no drug were considered as the 100 % benchmark of amastigote replication and the IC_{50} was calculated as the 50%of replication inhibition compared with the benchmark. Benznidazole, the current available drug, was used as positive control drug.

Unspecific mammalian cytotoxicity

J-774.1 murine macrophage-like cells (ATCC, USA) were maintained by passage in Dulbecco's modified Eagle's milieu (DMEM) containing 4 mM *L*-glutamine, and supplemented with 10 % heat-inactivated fetal calf serum. J-774.1 cells were seeded (1×10^5 cells/well) in 96 well microplates with 200 mL of RPMI 1640 milieu supplemented with 20 % heat inactivated fetal calf serum. Cells were allowed to attach for 48 h in a humidified 5 % CO_2 /95 % air atmosphere at 37 °C and, then, exposed to extracts for 48 h. Afterwards, cell viability was assessed by measuring the mitochondrial-dependent reduction of MTT to formazan. For this purpose, MTT was added to cells to a final concentration of 0.4 mg/mL and cells were incubated at 37 °C for 3 h. After removing the media, formazan crystals were dissolved in DMSO (180 mL), and the absorbance at 595 nm was read using a microplate spectrophotometer. Results were expressed as IC_{50} (extract concentration that reduce 50% control absorbance at 595 nm). Every IC_{50} was the average of five different experiments [12].

Nuclear Magnetic Resonance

Nuclear Magnetic Resonance spectroscopy was performed using the parameters explained by Kim et al. [32]. Ten mg of each extract were dissolved in 0.6 mL of CD_3OD + 0.15 mL of CD_3OD with 0.05 % of TMS. One dimension ^1H NMR spectra, ^1H-^1H homonuclear and inverse detected ^1H-^{13}C correlation experiments were recorded on Bruker DPX-400 spectrometer at 22.16 °C operating at a proton NMR frequency of 400.13 MHz. Methanol-d_4 was used as the internal lock. Each ^1H NMR spectrum consisted of 64 scans requiring 10 min and 26 s acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 ms), and relaxation delay (RD) = 1.5 ms. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra

were manually phased and baseline corrected, and referenced to internal standard TMS at 0.0 ppm, using Mestre Nova software version 6.0.

Data analysis and statistics

The ^{1}H NMR spectra were automatically reduced to ASCII files. Spectral intensities were scaled to internal standard and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.0–10.0 by AMIX software. The regions of δ 4.85-4.95 ppm and δ 3.2-3.4 ppm were excluded from the analysis because of the residual signal of H_2O and CH_3OH, respectively. Partial Least Square-Discriminant Analysis (PLS-DA) was performed with the SIMCA-P + software (v.12.0, Umetrics, Umea, Sweden).

Conclusions

Relevant biological activities against epimastigotes and amastigotes of *T.cruzi* were found for *Baccharis* genus *spp.* Collected in different Uruguayan locations and seasons. Also, all of them presented good selectivity against the parasite. The active principles, aldehyde-diterpenes, were identified by ^{1}H NMR-based metabolomics using the information obtained in the biological assays. The obtained results positioned *B. trimera, B. articulata* and *B. usterii* as candidates for new treatments of Chagas disease. Further complete characterization of the active principles and in vivo studies are currently being performed. Additionally from a phytomedicinal point of view we can infer that these extracts open a new vision in the development of safe and low cost drugs to treat Chagas disease.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank Eduardo Alonso Paz for his collaboration in the Botanical Classification of the plants used in this work. JV thanks ANII and CAP-UdelaR for his fellowships. This work was supported by project CSIC-UdelaR Nº612. Dr. A. Coqueiro thanks Ciências sem fronteiras, CAPES Foundation, Ministry of Education of Brazil, for the scholarship.

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