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Slowed Development of Natural Products for Chagas Disease, how to Move Forward?

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Abstract

Chagas disease, caused by *Trypanosoma cruzi*, is considered an endemic disease that affects millions of people causing generating health, economic and social problems. This study provides a review on research and development of new therapies for Chagas based in natural products of plant origin. We observed that there are more than 400 plant species that have been evaluated against different models of Chagas disease, and in some cases, there are interesting results. Challenge that hinders research work is the purification of the active compound and standardization of the chemical profile of whole extracts. The principal common factor that delays clinical testing is the lack of investment for the development of these products at the clinical phase. In the search of a natural, low cost and available drug for Chagas disease, we propose the use of new methodologies to overcome the existing challenges. The use of plant metabolomic technique is proposed as an option with high potential for the identification of biomarkers that could allow the standardization of chemical profiles. Furthermore, we describe the importance of applying good agricultural and manufacturing practices for reaching a successful development of quality phytotherapeutic products.

Keywords: natural products, metabolomics, *Trypanosoma cruzi*, Chagas disease

1. Introduction

“It does not explode like bombs or sound like shots. As hunger kills silently. As hunger kills the quiet, those who live condemned silence and die doomed to oblivion. The tragedy that does not ring, sick who do not pay, a disease that does not sell. Chagas disease is not a business that appeals to the pharmaceutical industry, nor is it a matter of interest to politicians

or journalists. Choose your victims in the pit. He bites them and slowly, little by little, and he ends up with them. Their victims have no rights, no money to buy the rights they do not have. They do not even have the right to know what they die from." *Informe clínico*, by Uruguayan writer Eduardo Galeano, in "*Chagas, una tragedia silenciosa*," Médicos Sin Fronteras, Editorial Losada, 2005.

Chagas disease is caused by *Trypanosoma cruzi*, transmitted to humans through the bite of *Triatomine* spp. by contaminated defecation rubbed on the mucosa. Transmission can also occur by ingestion of contaminated food, through blood transfusion or vertical transmission from mother to child [1]. It is worth to highlight that this endemic disease causes health, economic, and social problems affecting 6–7 million people in 21 countries from Central and South America. Moreover, 25 million people remain under risk of infection, given the high levels of population mobility from Latin America to the rest of the world or residence in endemic regions [2, 3].

Trypanosoma cruzi (*T. cruzi*) presents a quite complex cycle with different forms throughout its evolutionary cycle. In the insect vector, different stages can be observed. The *spheromastigote*, characterized as the round form of the organism, is predominantly found in the stomach of the organism. The epimastigote stage is found in the gut of the vector, at this stage, the *T. cruzi* intensively multiplies by binary division. And finally the metacyclic trypomastigote stage, which is the infecting form for the vertebrate host [4, 5]. When the insect feeds on human blood, it defecates on the host's skin, releasing into the feces parasites in the metacyclic trypomastigote form. Upon entry to the organ, trypanosomes are disseminated via the blood or lymphatic, affecting various organs mainly the heart, nervous system, muscle, and digestive system. Once the parasites reach the tissues they reproduce, passing through a non-flagellated stage, called amastigote, which is able to actively multiply themselves forming pseudocysts, where they are to be transformed into trypomastigotes that are later to be released [6]. It is estimated that the incubation period of the disease is between 5 and 12 days relative to each case, as well as the appearance of symptoms and the intensity of them. Often, cases are asymptomatic so timely diagnosis can be very difficult. Undiagnosed individuals may lead to conditions like serious cardiac and digestive conditions begin to arise that may lead to death [7]. It is possible to observe three well-defined stages of the disease. The initial stage is called acute or initial, which lasts approximately 2–4 months after infection. At this stage, many people do not have symptoms. Most patients with symptoms suffer from variable fever, malaise, irritability, headache, enlargement of the liver, spleen, and lymph nodes. When the inoculation is close to the ocular area, the characteristic chagoma is observed as well as a unilateral edema of both eyelids [8]. At this stage, the parasite can be found in the blood. Some acute cases can become deadly and many of these turn out to be young children and patients, who are immunocompromised (e.g. people infected with HIV), who may develop acute myocarditis or meningoencephalitis [9]. Then a second indeterminate or also called latent stage in which the infection becomes undetectable and is usually asymptomatic. It usually occurs between week 8 and 10 of the acute phase and it can last for months or even years. It is estimated that approximately 30% of individuals who reach this stage develop digestive, cardiac, and neurological problems [10]. Finally, the chronic phase which appears in the latter part of the infection, it is characterized by cardiac and intestinal problems. Sudden death can occur without the individual developing heart problems [11].

The diagnosis of this disease is relative to infection of the patient. Direct methods (direct microscopic observation, xenodiagnosis, PCR, etc.) based on the detection of genetic material or parasites or indirect methods (ELISA, IFI, Western blot, etc.) based on the detection of specific antibodies against *T. cruzi*, mainly used in the chronic stage, either symptomatic or asymptomatic. These are usually used mainly in the acute phase, in the case of immunosuppressed persons or under 6 months of age [12].

Unfortunately, an effective chemotherapy for all the clinical forms of the disease has not been reached, although fair enough time has passed by since the discovery of the disease in 1909 by a Brazilian Doctor, Carlos Ribeiro Justiniano Chagas [2]. This intractable situation is the typical case of neglected diseases, where the lack of adequate therapies or effective vaccines provoke health crisis [13]. Despite the revealing evidence of the current situation has not received sufficient attention from the pharmaceutical industry, mainly due to economic considerations. The current pharmacological treatments are based on two nitroheterocycles compounds, which were discovered more than 30 years ago: nifurtimox (Nfx, N-(3-methyl-1,1-dioxo-1,4-thiazinan-4-yl)-1-(5-nitro-2-methylpropan-2-yl)acetamide, Lampit®, suspended production, and sale by Bayer), and benznidazole (Bnz, N-benzyl-2-(2-nitroimidazol-1-yl)acetamide, Rochagan®, Roche, and currently produced By LAFEPE in Brazil). The problem with these drugs is that the significant side effects including weight loss, nausea and vomiting, rash, tissue abnormalities, leukopenia, neurotoxicity, psychosis, and peripheral neuropathy. On the other hand, Bnz can cause edema, fever, rash, peripheral neuropathy, lymphadenopathy, agranulocytosis, thrombocytopenic purpura, and joint and muscular pain. The use of these drugs during the acute phase of the disease is widely accepted, but its efficacy in the chronic phase is controversial [14].

The medicinal chemistry of Chagas disease has used different approaches in the search for new therapeutic entities. Some are oriented to the chemical development (synthesis) of new agents with interaction with key biomolecules for the parasite, or production of toxic species [15, 16]. Also, the reposition and polypharmacology of drugs have been used [17]. Other strategies have been oriented to the identification and isolation of new agents of natural origin [18]. Despite these efforts, research work in this medical area in order to find new solutions to a problem that seems to have no end is therefore, of utmost importance [14]. This review seeks answers in nature and always remembers the primary motivation: that affected people lacking the necessary resources find in their natural habitat medicinal plants that provide a possible treatment endorsed by science, effective and without side effects.

2. Natural products research

The use of plants for curative purposes dates back to the beginning of human history. The man turned to nature in search of food and health. By means of successes and errors, he learned to know the plants that healed. This knowledge was transmitted from generation to generation and was increased with experimentation. Without the resources offered by nature, humans would not have survived. Gradually humans, by dominating nature, have broken

many of the ties that bind him. Today the medicine uses synthetic or semisynthetic drugs to relieve all diseases. Many of these drugs are beneficial, but many also, by misuse or abuse have lost their efficacy and in countless cases cause harmful side effects [19, 20].

In Latin American countries, the use of medicinal plants is the usual practice of indigenous groups and is frequently used by certain sectors of society [21]. This practice is usually an economic alternative to the prices imposed by the pharmaceutical industries and in many cases the only possibility of treatment [21, 22]. Most medicinal plants have multiple physiological effects, due to the presence of more than one active principle. The latter correspond to chemical compounds of the plant, which are subject to variables, such as soil moisture, light conditions, temperature, date of planting and harvesting, drying conditions, and among others [23].

Fortunately, in recent years, there has been an increase in interest in the return to nature, and therefore, it is necessary to build a new relationship with our environment, leading a less artificial life and turning to plants not only to include them in our diet but also to alleviate our conditions [19].

Natural products contribute greatly to the history and landscape of new molecular entities (NMEs). An assessment of all FDA-approved NMEs reveals that natural products and their derivatives represent over one-third of all NMEs. By the end of 2013, the FDA had approved 547 natural products and derivatives. Since the 1970s, the relative and then an absolute number of natural-product-based NMEs began to decline and today stand at fewer than one-quarter (24%) or an average of 7.7 natural product NMEs per year [24]. Plant products represented more than one-fifth (22%) of all NMEs approved before 1950, declining by more than 50–8.7% since that time [24]. Over the past two decades, the pharmaceutical industry has shied away from research into natural products. Attention shifted toward combinatorial chemistry, which seemed to satisfy the need for compound libraries to keep up with high throughput assays based on newly discovered molecular targets. However, this approach did not result in improved productivity, nor did an increase in the number of new drugs. The number of NMEs reached a record low of 24 in 2004 with an average of 40–50 new approved drugs by year in the period of 1981–2014. In the same period of 1981–2014, 16 NMEs were approved as antiparasitic drugs. Newman and Cragg classified these NMEs as unaltered natural products, natural products derivatives, a synthetic drug, synthetic drug (natural product pharmacophore), and mimic of the natural product (**Figure 1**). Among them, 68.7% are related to natural products [25, 26].

Izumi et al. reviewed the prospect of developing new drugs for the Chagas disease, on the screening of almost 400 species belonging to more than 100 plant families for activity against *T. cruzi* [27]. The plant extracts preparation methods have been very variable, from processes involving only one part of the plant to extract with different polarity solvents as well as using all part of the plant in the same solvent. Usually, the plant part in the study is the same that is used traditionally, but this does not assure that the accumulation of the active principles is maximum in the selected part. The plant extracts screened against different intracellular

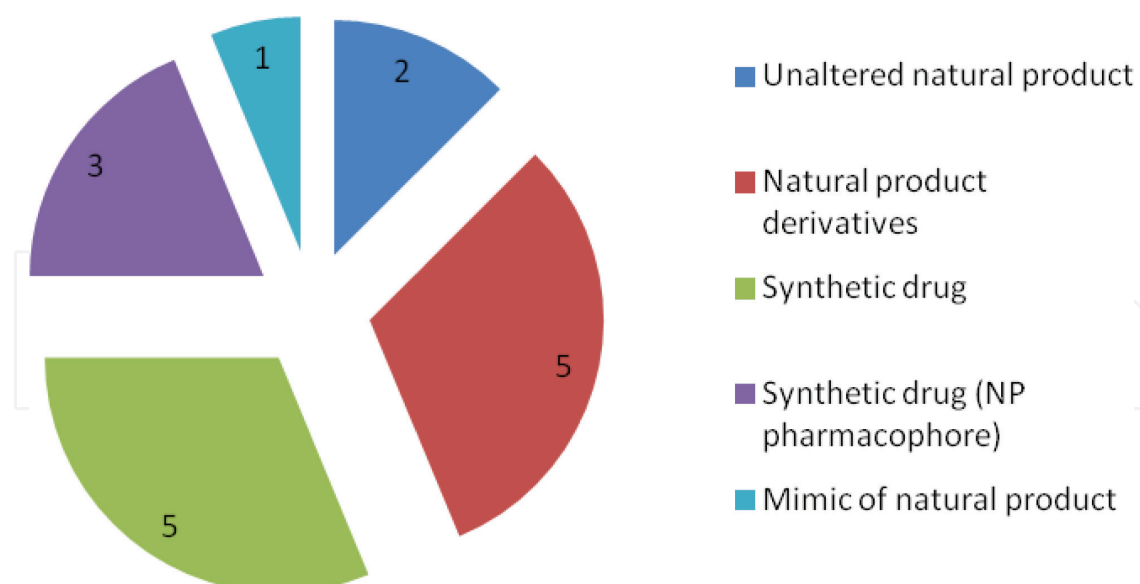


Figure 1. Classification of antiparasitic NMEs approved by FDA in the period 1981–2014.

forms of *T. cruzi* shows promising results. As a result, hexane extracts of *Polygala sabulosa* and aqueous extracts of *Polygala cyparissias* showed 50% inhibition of epimastigote growth after 72 h of treatment at concentrations of 1 and 2 $\mu\text{g/mL}$, respectively [28]. Ethanol extracts of *Physalis angulata* showed 50% inhibition of epimastigote growth after 120 h of treatment at a concentration of 2.9 $\mu\text{g/mL}$ in Y strain and 7.4 $\mu\text{g/mL}$ in Colombian strain [29]. Ethanol extracts of *Baccharis trimera* and *Baccharis articulata* showed 50% inhibition of epimastigote growth after 120 h of treatment at concentration 13.6 and 16.6 $\mu\text{g/mL}$ in Tulahuen 2 strain, respectively [30]. For trypomastigotes, *Piptadenia africana* methanolic extract caused lysis in 50% of the parasites at 4 $\mu\text{g/mL}$ after 96 h [31], and a methanolic extract from *Gardenia lutea* also promoted the same effect at approximately 22 $\mu\text{g/mL}$ after 72 h [32]. The essential oil from the fruits of *Piper cubeba* showed 50% inhibition of trypomastigote form at a concentration of 45.5 $\mu\text{g/mL}$ [33]. Surprisingly, by applying methanolic extracts of eight different species (*Hypoestes forsskalii*, *Kleinia odora* and *Psiadia punctulata*, *Capparis spinosa*, *Euphorbia schimperiana* and *Ricinus communis*, *Marrubium vulgare*, and *Solanum villosum*) an inhibition of 50% less than 0.25 $\mu\text{g/mL}$ was found against the amastigote form with a treatment of 7 days [34]. Also for amastigotes, methylene chloride extract from leaves of *Conoclea scoparioides* showed 50% inhibition after 96 h at 1.3 $\mu\text{g/mL}$ [35]. Ethanol extracts of *Baccharis trimera* and *Baccharis articulata* showed 50% inhibition of amastigote growth after 72 h of treatment at concentration 9.9 and 22.3 $\mu\text{g/mL}$ in Dm28c strain, respectively [30]. Given that the financial resources for research on neglected diseases are usually low, most of the laboratories cannot afford the maintenance of infected animal models or cell cultures to perform tests with the infective forms, trypomastigote, and amastigote. This leads to screenings of compounds performed only against non-infective forms, even when promising preliminary results are reached, the isolation of active compounds is not frequent. Despite the screening of hundreds

of species, the major, possibly active compound was only isolated, identified, and evaluated for antiparasitic activity in approximately 10% of the cases [27]. *In vivo* studies on the animal model of Chagas disease of plant extracts are less numerous than *in vitro* ones, but there are also reports of promising results. For example, *Serjania yucatanensis* ethanol leaves extract reduced 75% of the parasitemia in infected mice treatment at 100 mg/kg [36] and *Aristeguietia glutinosa* ethanol aerial parts extract reduced 50% of the parasitemia in infected mice at 50 mg/kg [37]. Plants extracts screening performed have been based on isolated efforts directed by research groups worldwide. Moreover, Pereira et al., after conducting an extensive bibliographic review for natural antichagasic products, conclude that there is an important geographic and ethnodirected component in the research initiatives on the relevance of plant derivatives in the treatment of Chagas disease [38]. The main obstacle that stops research work is the gap that needs to be bridged toward clinical phases, given the difficulties in scaling the purification of the active compound, standardization of the chemical profile of whole extracts, and among others. The principal common factor that delays clinical phase is the lack of investment for the development of these products at this stage of investigation [39]. **Figure 2** summarize the progress and current status of research and development of natural products for the Chagas disease.

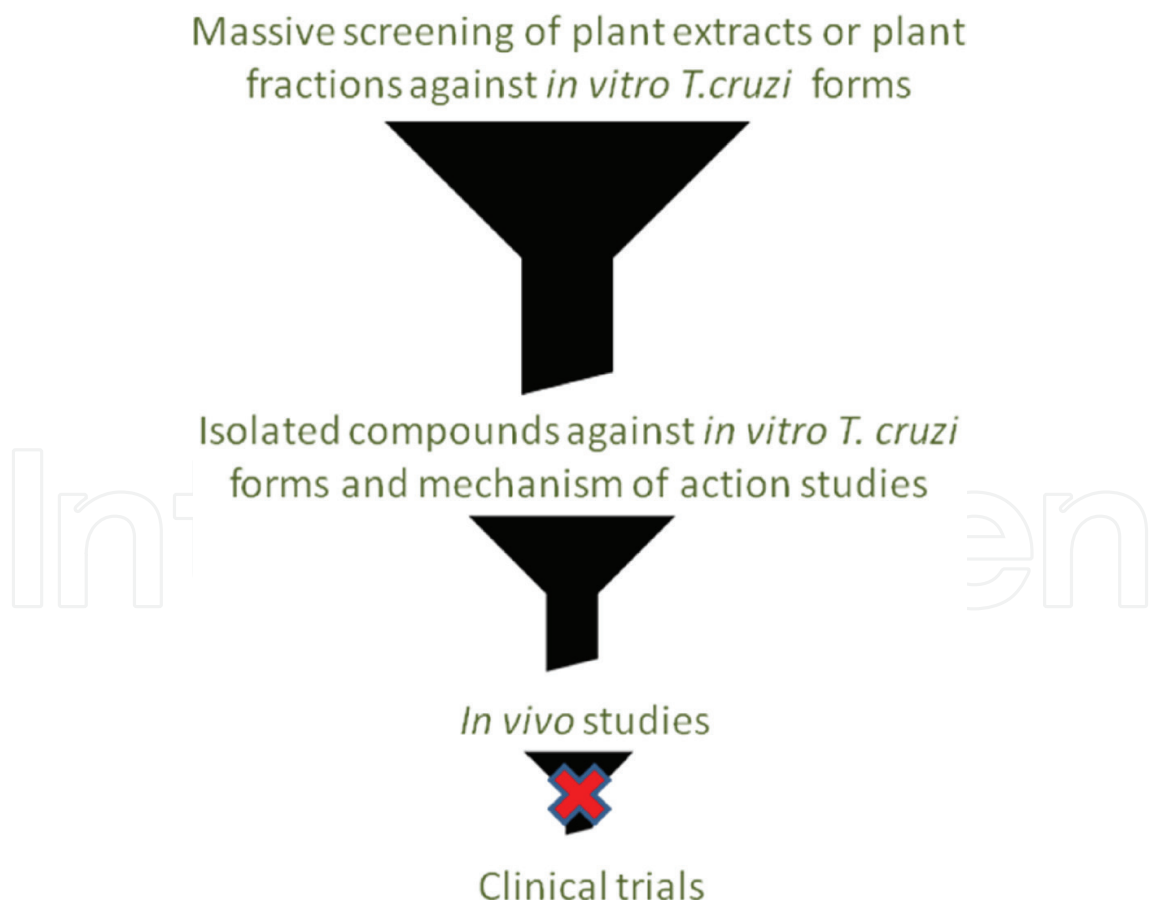


Figure 2. Current status of research and development of natural products for the Chagas disease.

In many cases, the isolation of compounds leads to the loss of biological activity or to the achievement of a really low extraction yield. That is why work with whole extracts should be re-considered as a possible source of new treatment drugs for the Chagas disease. Given the low economic potential of the majority of the affected population, it is clear that the development of new treatment drugs should consider cost-effectiveness. In the search for new therapies for Chagas from plant origin, low cost and easy access for patients, we propose the use of new methodologies to overcome the existing challenges. The use of plant metabolomics technique is proposed as an option with high potential for the identification of biomarkers that could allow the standardization of chemical profiles of whole plant extracts that can be formulated in a simple, quick, and low-cost dosage.

3. Plant metabolomics

Metabolomics can be defined as the detection and quantification of all metabolites of low molecular weight in an organism at a given time and in certain conditions. However, adjusting to our purposes and applications can be better defined as the area of research that seeks to obtain the metabolic fingerprints to detect the differences between them and propose hypotheses that explain the differences.

The field of metabolomics in science marks its beginning when Devaux and Horning publish their research work in the metabolic profile where they apply gas chromatography coupled with mass spectrometry (GC/MS) for the analysis of extracts of human tissues and urine [40]. Immediately, the interest of different groups in using the metabolic profiles for the diagnosis and follow-up of different pathologies [41]. During the 1970s metabolomics studies expanded to a wide range of activities including: novel techniques for detection and elucidation of insect hormones [42], identification of natural products of marine origin [43], and chemotherapeutic agents derived from plant extracts (e.g.: *Hyptis tomentosa*) [44]. At the beginning of the 1980s, the first work on automated metabolic analysis emerged [45] and by the middle of the decade, the first works on metabolic profiling were published using nuclear magnetic resonance (NMR) and high-performance liquid chromatography (HPLC) [46–48]. In order to analyze the mechanism of action of herbicides besides performing GC/MS, a new global approach was developed in 1991 [49]. As a result of scientific cooperation, by the end of twentieth century, it was possible to apply novel technologies to metabolomics and methods of extraction, encouraging the development of metabolite databases [50], which allowed the global development of complete metabolomes [51].

In the last decade, metabolomics has developed as an important field within plant science and natural product chemistry [52–56]. Metabolic fingerprint, also known as a metabolic profile, is an objective analytical approach that seeks to quantify a group or groups of compounds found in an organism or group of organisms. The metabolic profile with gas chromatography and high-performance chromatography coupled to mass spectrometry or proton magnetic resonance (^1H NMR) has been successfully used the study plant biochemistry,

chemotaxonomy, ecology, pharmacology, and quality control of medicinal plants [57, 58]. The application of NMR has already been demonstrated as a suitable and sufficient method to carry out this type of analysis, since it allows simultaneous detection of various groups of secondary metabolites, in addition to abundant primary metabolites [59]. In addition, ^1H NMR spectroscopy has a great advantage over the other techniques, the signal intensity is dependent only on the molar concentration of the metabolites, allowing the direct comparison of these present in the sample [60, 61]. In the last years, several reports on the evaluation of the metabolic differences in *Cannabis sativa*, *Vanilla planifolia*, *Vitis* spp. and *Catharanthus roseus*, among others, and in the classification of *Ilex* species based on their metabolome are published [57, 60, 62, 63]. The major disadvantage in using NMR spectroscopy in the metabolomic analysis is overlapping signals, which however, can be solved by using different 2D-NMR techniques [52, 60, 63].

A typical approach of NMR-based metabolomic application for the identification of new natural products with biological activity that are part of whole extracts (**Figure 3**) was applied for the identification of new natural products with anti-*T. cruzi* activity in Uruguayan plants. Eighty samples of ethanolic extracts from different botanic parts, soils, and seasons, of Uruguayan specimens: *Baccharis trimera*, *Baccharis articulata*, *Baccharis usterii*, *Hydrocotyle bonariensis*,

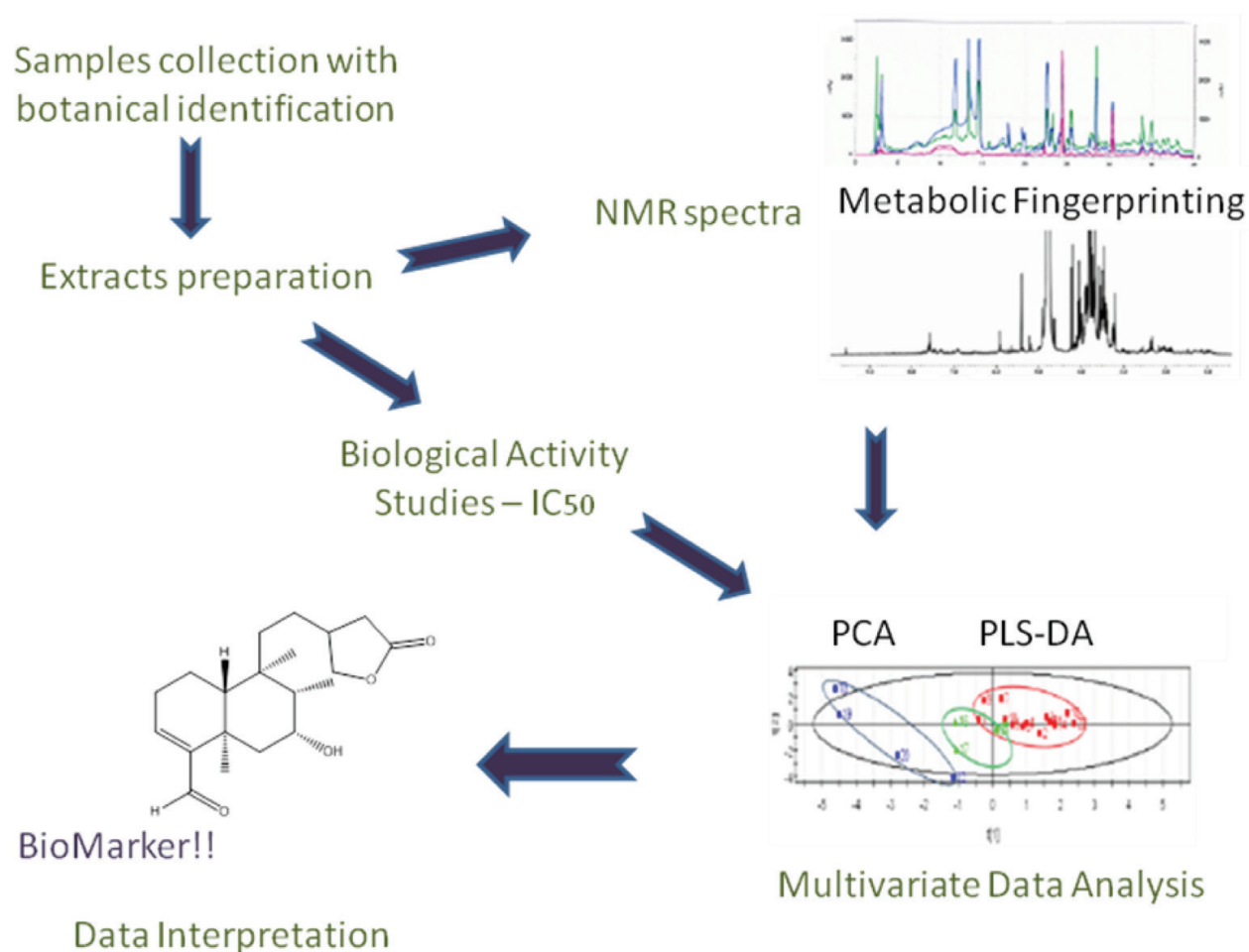


Figure 3. Typical approach of NMR-based metabolomic to identify new active compounds on plant extracts.

Achyrocline satureioides, *Taraxacum officinalis*, and *Plantago major* were used. As a primary screening the anti-*T. cruzi* activity against the epimastigotes showed that three species of *Baccharis* genus and *Hydrocotyle bonariensis* displayed well to excellent antiproliferative activity. The most active fractions were additionally evaluated for their activity on amastigotes and also for cytotoxicity against mammalian cells. For the identification of the active principles was applied using nuclear magnetic resonance-based metabolomic. Through the metabolomic study of the relationship between the changes in chemical profiles and the biological activities, it was possible to identify the main active principles of the extracts and also the compounds responsible for cytotoxic activity [30]. The technique also allowed us to infer the parts of the plants with greater accumulation of the active compounds as well as the conditions for their maximum expression (soil type, harvest season).

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. We performed the standardization of cultivars of *Baccharis trimera*, *Baccharis articulata*, and *Baccharis usterii* with relevant results in the expression of their active molecules against *T. cruzi*. The propagation was made by cloning mother plants collected from wild nature and developing their cuttings on an environment controlled greenhouse (Figure 4). The plant extracts prepared from the standardized cultivars are being studied *in vivo* in a murine model of the Chagas disease.

When plant cultivars are performed for possible pharmaceutical uses it is important to consider World Health Organization (WHO) guidelines on good agricultural and collection practices (GACP) for medicinal plants [64].



Figure 4. Cultivar propagation of *Baccharis* spp. for standardization of their chemical profile for extracts preparation to be used on *in vivo* test for the Chagas disease.

4. Good agricultural and collection practices (GACP)

Medicinal plant resources are being harvested in increasing volumes, largely from wild populations. Indeed, demand for wild resources has increased by 8–15% per year in Europe, North America, and Asia in recent decades. Various sets of recommendations relating to the conservation of medicinal plants have been developed, such as providing both *in situ* and *ex situ* conservation [65].

The harvest of plants that had a wild growth is considered an efficacious source for medicinal purposes, but domestic cultivation is also a widely used and accepted practice [66–68]. Indeed, domestic cultivation provides advantages for the production of medicinal plants, allowing control for toxic components, avoiding pesticides, increasing the content of active compounds, and having precise information for the identification of botanical origin [69]. In this way, controlling the growing conditions is a relevant tool for production stability and to improve the yields of secondary metabolites, which are frequently the active compounds. Cultivation standards include providing optimal levels of water, nutrients and other environmental factors such as light, humidity, temperature, in order to improved yields of target products [70]. Moreover, controlled cultivation contributes to decrease the harvest of medicinal wild plant resources, also having a positive impact on their prices [71, 72].

This knowledge has been translated into good agricultural and collection practices for medicinal plants that were developed in order to regulate production, assess quality, and lead to the standardization of herbal drugs [73]. The application of these formal practices, summarized in GACP approaches, is important to ensure high quality, safe and pollution free herbal drugs (or crude drugs) [74]. A wide range of problems are controlled by applying GACP including the ecological environment of production, germplasm, cultivation, and collection methods, as well as quality aspects for pesticide detection, authentication in macro and microscopic terms, chemical identification of active compounds, and detection of metal elements [75]. Given the importance of GACP, many countries actively promote their implementation; however, there is still an important gap to bridge concerning knowledge and implementation. This disparity is given by the difficulties encountered for training farmers and other relevant actors for medicinal plant production, such as handlers and processors. While these kind practices are strictly applied at the level of pharmaceutical producers that are used to work in order to meet quality control requirements, it has been more difficult to successfully introduce this practices standard at the level of agricultural producers, handlers, and processors of medicinal plant material. It will be important to focus the efforts on training farmers and other relevant actors to ensure that GACP is adopted and favor the obtainment of high-quality medicinal plant materials [64].

Currently, organic farming is increasingly receiving public attention, given that these practices include a vision of sustainability and economically relevant business without forgetting the well-being of workers and creates integrated production systems for medicinal plants [76, 77]. The main characteristic of organic farming is the avoidance of synthetic fertilizers, pesticides or herbicides, and reaching the standards of organic certification. The defining characteristic of organic farming is the non-use of synthetic fertilizers, pesticides, and herbicides, which are

not allowed according to many current organic certification standards in Europe and North America. Instead of applying synthetic fertilizers, organic fertilizers may be continuously supplied to the soil, contributing nutrients, and improving soil stability, while positively impacting the biosynthesis of essential substances. Furthermore, organic farming generates high-quality products and better yields, while taking care of the conservation of those plants. Indeed, when organic fertilizers were applied to the cultivation of *Chrysanthemum balsamita*, the biomass yield was increased and its essential oil content was higher than those free from organic fertilizers [78]. Above all, organic farming is a benign practice for our environment, based upon renewable and sustainable resources that favor the maintenance of a biological equilibrium in the medicinal plants and their ecological systems [74, 76]. For these reasons, the application of organic farming practices is highly relevant for medicinal plant production, encouraging a sustainable, and long-term systemic approach [77].

5. The novel strategy proposed to obtain effective, cheap and standardized phytoterapeutic for treat Chagas disease

Based on the use of metabolomic and the GACP, the following workflow is proposed for obtaining phytoterapeutics for Chagas disease treatment (Figure 5).

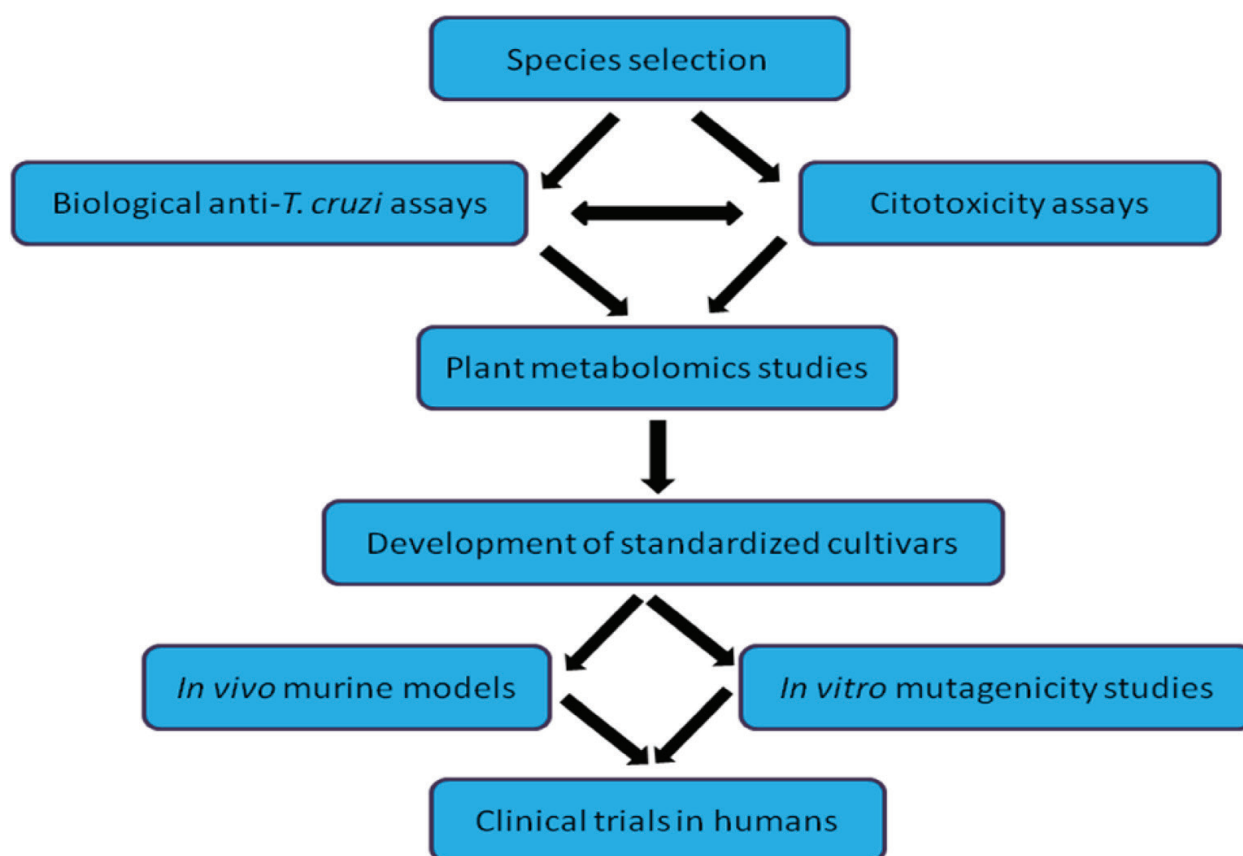


Figure 5. Workflow proposed for research and development of new natural products for Chagas disease treatment.

6. Conclusions

Traditional medicines, particularly herbal medicines have been increasingly used worldwide during the last two decades. On Chagas disease research many plant extracts were evaluated worldwide with relevant results but in most cases, the development is slowed in the scaling of the isolation of the active principle or by the lack of standardization of chemical profiles of the whole extracts. We presented a new novel strategy for the production of effective, cheap and standardized phytotherapeutic products through the use of plant metabolomics technique and the standardization of medicinal plants cultivars applying good agricultural and collection practices.

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