

Anti-Phytomonas activity of the lyophilized residues obtained from the distillation of Lantana camara L. essential oil

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Abstract

On an industrial scale, the residues accumulated in essential oil distilleries can be compared to the volume of residues produced in a textile industry. Although these residues are discarded, they possess molecules with diverse biological activities, including their application in phytopathogen control. In this study, the chemical profile of the residue from the hydrodistillation of *Lantana camara* L. leaves was determined using High-Performance Liquid Chromatography (HPLC). Additionally, the effect of the residue on cells was assessed by determining plasma membrane integrity, levels of reactive oxygen species (ROS) production, and mitochondrial potential depolarization. The viability and cell density of *Phytomonas serpens* parasites significantly decreased after treatment with increasing concentrations of the lyophilized residue. RL038, the lyophilized residue from accession LAC-038, reduced cell viability by an average of 61.36%. ROS levels increased by approximately 2x and 3x at RL038 concentrations of 120 µg/mL and 180 µg/mL, respectively. It was observed that the same concentrations modified mitochondrial potential, reducing fluorescence by 44.6% and 46.8%, respectively. Analytical liquid chromatography of RL038 revealed the presence of 17 peaks subsequently classified as phenolic acids and flavonoids. RL038 from the hydrodistillation of *Lantana camara* L. leaves is a source of biologically active compounds with antiprotozoal potential.

1. Introduction

Essential oils are mixtures that are rich in aromatic compounds and they have several applications. They are currently used as ingredients in the production of beverages, food, fragrances, cosmetics, and drugs, in addition to being considered the primary tool of aromatherapy, a segment of medicine in which the treatment of patients occurs with the help of these oils (Shankar et al., 2021). Although there is a growing demand for these products (Alighiri et al., 2017), the yield of essential oil in many plant species is low. This results in a large volume of residues during the distillation process (Napoli et al., 2020). On an industrial scale, the waste that is accumulated in the distilleries of essential oils can be compared to the volume of waste that is produced by the textile industry (Gharred et al., 2020). The incorrect disposal of these materials can cause severe damage to the environment.

The maximum use of raw materials, the reduction of energy expenditure and waste production, as well as their transformation into new products, is part of the biorefinery concept. In the essential oils industry, the residues are generally discarded. However, several useful compounds can be extracted from them in other production chains, such as rosmarinic acid, an important natural antioxidant that can be obtained from the liquid residue of the hydrodistillation process of the leaves of rosemary (*Rosmarinus officinalis* L.) (Wollinger et al., 2016). This is together with the phenolic compounds that are extracted from the solid residue of the hydrodistillation of *R. officinalis*, which have a deterrent effect on pest insects (Sánchez-Vioque et al., 2015). Sankara et al. (2020) reported that the residues from the hydrodistillation of *Eucalyptus camaldulensis* could provide interesting bioactive compounds, as new anti-termite agents in plant protection. Mahmoudi et al. (2020) also reported that hydrodistilled basil leaves (*Ocimum basilicum*) have a good repellent activity against the stored grain pests, *Tribolium castaneum*.

Lantana camara L. (Verbenaceae) is an aromatic species widely known throughout the world for its ornamental use. In traditional medicine, it is common to use its leaves, branches, and roots to treat cuts, fevers, headaches, palpitations, tetanus, rheumatism, malaria, ulcers, and so forth. (Gindri and Coelho, 2020). In the essential oil that is obtained from this species, several bioactive compounds are found, such as terpenoids, alkaloids, flavonoids, phenolic compounds, and glycosides. These compounds are to which various biological activities have been attributed, such as anticancer and antiangiogenic (Al-Hakeim et al., 2021), anti-inflammatory (Wu et al., 2020), anti-tuberculosis (Nirmal et al., 2020), as well as a sedative (Dougnon, Ito, 2020). This is in addition to antifungal (Bashir et al., 2019), herbicide (Qureshi, et al., 2019), nematicide (Borbodoli et al., 2021), insecticide (Ayalew, 2020), and trypanocide agents (Delgado-Altamirano et al., 2019).

Trypanosomatids are microorganisms that are adapted to life in fluids that are rich in glucose, where most of them are parasites of animals, with the exception of the genus *Phytomonas*, whose representatives are plant parasites (Maslov et al., 2019). Some species cause serious diseases in large crops of economic interest. *Phytomonas staheli*, which causes acute lethal wilt in coconut palms (*Cocos nucifera*), and oil palm (*Elaeis guineensis*), which begins with the rotting of the leaves, and then progresses to the spear and the roots, as well as *Phytomonas leptovasorum*, a pathogen of necrosis in coffee (Arabica and Liberia) phloem (Jaskowska et al., 2015). These are some examples of the important plant pathogens, which are capable of offering an economic risk in tropical countries.

Phytomonas serpens is not a pathogen of agricultural relevance, although it is an important biological model that helps in the understanding of pathogenic members belonging to the Trypanosomatidae family that affect plants and mammals. Due to its simplified biology, it helps in understanding the mechanisms of pathogenicity and the manifestations of diseases in plants and animals (Jaskowska et al., 2015, Santos Junior et al., 2018).

In a recent study, this current group demonstrated for the first time, the trypanocidal activity of the essential oil of *L. camara* on *P. serpens* (Pereira et al., 2022), demonstrating the potential of this product for the treatment of economically important diseases that are caused by other species of *Phytomonas*. However, the yield of *L. camara* essential oil is low (usually 0.2%), and by steam dragging, 500 kg of biomass yields about 200 mg of oil (Negi et al., 2019). On the other hand, the volume of waste generated by the oil hydrodistillation process is high and constitutes a promising source of bioactive molecules.

Given the commercial importance of crops that are devastated by species of the genus *Phytomonas*, and the potential of the products in the metabolism of *L. camara* to control the proliferation of these phytopathogens, the present work evaluated the action of residues in the hydrodistillation of the leaves of *L. camara* on the viability of *P. serpens in vitro*. This was as well as their effects on the integrity of the plasma membrane, and the potential of the mitochondrial membrane on these phytopathogens.

2. Material and Methods

2.1 Plant material

Four accessions of *Lantana camara* L., LAC-033, LAC-038, LAC-017, and LAC-025, from the Active Germplasm Bank of Medicinal and Aromatic Plants of the Federal University of Sergipe, Brazil, were selected. The choice of the accessions was supported by previous works, in which the chemical characterization of the essential oils from the plants of the active germplasm bank was determined (Pereira et al., 2020). The accessions of the plant material were registered under the identification number A8CCB3B in the National System for the Management of Genetic Heritage and Traditional Knowledge (SisGen).

The leaves were collected and subjected to hydrodistillation in a modified Clevenger apparatus, by using 75g samples of the dry leaves, with the addition of 1.5L of distilled water, for 140 minutes. The effluent, resulting from the decoction of the leaves, was filtered and separated into 200mL samples. Later, they were stored in an ultra-freezer at -80°C (Liotop UFR30, Liobras, São Carlos, SP, Brazil) for 24 hours. They were then transferred to a freeze dryer (Liotop L101, Liobras, São Carlos, SP, Brazil), where they rested for five days at a temperature of -54°C and a pressure of 79 $\mu\text{m Hg}$ until the complete sublimation of the water that was contained in the samples. At the end of this process, the “Lyophilized Residues (LRs)” were obtained from each accession, hereinafter named LR038 (Accession LAC-038), LR017 (Accession LAC-017), LR025 (Accession LAC-025), and LR033 (Accession LAC-033).

2.2 Cell culture

The *Phytomonas serpens* (9T) parasites were obtained from the Trypanosomatid Collection at the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. The promastigote forms were grown in a Schneider culture medium (Sigma-Aldrich®, St. Louis, Missouri, USA), which were supplemented with 10% fetal bovine serum (Sigma-Aldrich®, St. Louis, Missouri, USA) and 1% streptomycin at 26°C in a BOD incubator.

2.3 Cell viability of *Phytomonas serpens* when exposed to the residues from the *Lantana camara* accessions

The promastigotes were cultured at 26°C in a BOD incubator, and then 1×10^5 cells were deposited in 96-well microplates and treated for 48 hours, with the four lyophilized residues (LRs), in serial dilutions at concentrations of 100.0 to 1.5 $\mu\text{g/mL}$. The negative control consisted of the untreated cells, and for the positive control, the cells were treated with the antibiotic, Amphotericin B. After 48 hours of treatment, 50 μL of resazurin solution (200 μM) was added to each well, and the plates were incubated in the dark for 30 minutes. This particular dye is an indicator of cell viability, which when added to the culture, enters the cells and is reduced by the mitochondrial dehydrogenases to the resofurin of the viable cells while changing the color of the medium from blue to pink. The cell viability was measured by spectrofluorimetry at a wavelength of 555 nm excitation and 585 nm emission in a microplate reader (Synergy™ H1, BioTek Hybrid Technology, Winooski, Vermont, USA). From the cell viability values

obtained, the IC₅₀ levels (the concentration that inhibits 50% of the cells) were calculated. The lyophilized residue (LR) from the accessions that presented the lowest IC₅₀ value was used in the subsequent tests.

2.4 Growth curve of *Phytomonas serpens* when treated with selected residue

The promastigotes (1x10⁵ cells/mL) were treated or not with the lyophilized residue (LR), which had a lower IC₅₀ at the concentrations of 6.25, 12.5, 25.0, 50.0, and 100 µg/mL, and then incubated in 12-well plates for 72 hours, in a BOD oven at 26°C. The number of cells was evaluated at 24, 48, and 72 hours, by direct counting in a Neubauer chamber when under an optical microscope with a 10X lens. The results were used to construct the growth curve.

2.5 Effects of LR on the *Phytomonas serpens* cells

The effects of LR on the integrity of the plasma membrane, on the potential of the mitochondrial membrane, and on the production of the reactive oxygen species, were evaluated for the *P. serpens* cells. In all of the evaluations, the promastigotes (5.10⁵ cells/mL) were treated for 48 hours with the different concentrations of the selected lyophilized residue (LR). They were then used for the specific procedures for each assessment, as described below.

2.6 Plasma membrane integrity

The treated cells were exposed to propidium iodide (40 µg/ml) for 15 minutes. The incorporation of propidium iodide, which was indicative of the loss of membrane integrity, was evaluated by flow cytometry when using an Attune® Acoustic Focusing Cytometer (Life Technologies, Carlsbad, California, USA). A total of 10,000 events were obtained and analyzed by using the Attune® Cytometer Software program. The incorporation of propidium iodide was also evaluated by fluorescence spectrometry when using the wavelengths of 488 nm excitation and 620 nm emission. As a negative control, the untreated cells were used, and the positive control was by the cells that were heated at 80°C for 10 minutes.

2.7 Mitochondrial membrane potential

After the treatments, the cells were washed twice in sterile phosphate buffered saline (PBS) (3000 rpm / 10 minutes / 4°C) and were resuspended in 100 µL of PBS. The cells that were treated with LR were exposed to 10 µg/mL of Rhodamine 123 (Rh123, Sigma-Aldrich, St. Louis, Missouri, USA) in the absence of light for 15 minutes. After the treatment period, 400 µL of PBS was added, and the changes in the mitochondrial membrane potential were assessed by flow cytometry by using an Attune® Acoustic Focusing Cytometer (Life Technologies, Carlsbad, California, USA).

A total of 10,000 events were obtained and studied when using the Attune® Cytometer Software program. The cells that were not treated with LR were used as a negative control, and the cells that were treated with 50 µM of 4-chlorophenylhydrazine carbonyl cyanide (CCCP) were used as a positive control. The variation index (VI) was determined by the equation (MTC-MUC)/MUC, in which MTC corresponded

to the Rh123 fluorescence median of the cells that were treated with the LR, while MUC was the median fluorescence of the untreated cells.

2.8 Reactive oxygen species

After the treatments, a volume containing 1,107 promastigotes was washed twice in sterile PBS (3000 rpm / 10 minutes / 4°C) and later resuspended in 100 µL of PBS, and then transferred to 96-well microplates. The cells that were treated with LR were exposed to 20 µM 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA) in the absence of light for 30 minutes. The fluorescence produced by the formation of 2,7-dichlorofluorescein (DCF), as a result of the interaction with H₂DCFDA with the produced ROS, was measured by spectrofluorimetry at wavelengths of 492 nm excitation and 527 nm emission when using a Synergy™ H1 microplate reader (BioTek Hybrid Technology, Winooski, Vermont, USA). As a negative control, the untreated cells were used, and as a positive control, the cells that were treated with 750 µM of hydrogen peroxide (H₂O₂) for 30 minutes were used.

2.9 Qualitative chemical profile of the lyophilized residue (LR)

The chromatographic exploration to obtain the qualitative chemical profile of the lyophilized residue from the accessions of the *L. camara* collection that displayed a greater antiprotozoal activity was performed by a Shimadzu Prominence network-ready system for analytical liquid chromatography (Kyoto, Japan). This consisted of a CBM-20A communication module, a DGU-20A3 degasser, an LC-20AT binary pump system, a SIL-20AHT automatic injector, a spectrophotometric detector with an SPD-M20A diode array, and a CTO-20A column oven.

The analyzed samples were prepared by dissolving 10 mg of LR in 1 mL of ultrapure water (10 mg/mL), then strained through a 0.45 µm syringe filter, and transferred to the analysis channel. For the separation of the chemical compounds that were present in the samples, a C18 stationary phase chromatographic column (250 x 4.6mm, 5µm, 100 Å, Kinetex, Phenomenex, Torrance, California, USA) was used. This was connected to a guard column of the same stationary phase, with exploratory elution gradients of 5 to 100% B for 60 min, 100 to 100% B for 10 min, 100 to 5% B for 5 min, as well as with 40 minutes of conditioning the column to 5% B. The sample injection volume was 25 µL and the column temperature was 30°C. The mobile phase was formed by a binary mixture of methanol (B) and an aqueous solution of formic acid 0.5% (v/v) (A) at a flow rate of 1mL/minute. The wavelength used for the detection of the chemical compounds was 254 nm.

2.10 Data analysis

The data was subjected to an Analysis of Variance (ANOVA), while using Tukey as a post-test, with the aid of the GraphPad Prism 7.01 program. The results were expressed as mean ± standard error, and the differences concerning the untreated controls were considered significant when $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). All of the experiments were carried out in triplicate.

3. Results

3.1 Cell viability of *Phytomonas serpens* when exposed to the residues from the *Lantana camara* accessions

Initially, a screening of the antiprotozoal activities of the lyophilized residues (LR) of the four *L. camara* accessions on the cell viability of *P. serpens* was carried out. The parasite cell viability decreased as the LR concentrations increased (Fig. 1A). The IC₅₀ values that were obtained from the four LRs of *L. camara* were 13.68 µg/mL (LR038), 17.50 µg/ml (LR017), 35.16 µg/ml (LR025), and 43.69 µg/ml (LR033) (Fig. 1B). Although the four LRs showed good inhibition of the parasite viability, LR038 was selected for further experiments because of its lower IC₅₀.

3.2 Growth curve of *Phytomonas serpens* when treated with selected residue

LR038 affected the proliferation of *P. serpens* at all of the concentrations evaluated, and the concentrations of 50 µg/ml and 100 µg/m of LR038 markedly reduced the cell density of the promastigote forms (Fig. 2A). After 24 hours, the concentration of 50µg/mL reduced 47% of the cell growth and the concentration of 100µg/mL inhibited 59%. After 48 hours, the same concentrations reduced 52% and 76% of the growth of promastigotes, respectively. After 72 hours, there was a 65% reduction in the cell density at the 25 µg/mL concentration of LR038, and an 84% and 95% inhibition was observed at the 50 µg/mL and 100 µg/mL concentrations, respectively (Fig. 2B).

3.3 Evaluation of the cell membrane integrity of the *Phytomonas serpens* promastigotes

To determine the integrity of the membrane, a high molecular weight DNA intercalant that did not penetrate the cell with an intact membrane was used with the fluorescent probe, propidium iodide (PI). The flow cytometry and spectrofluorimetric analysis demonstrated that LR038 at the concentrations evaluated did not affect the integrity of the plasma membrane (data not shown).

3.4 Evaluation of the reactive oxygen species production of the *Phytomonas serpens* promastigotes

The production of the reactive oxygen species in the *P. serpens* promastigotes that were treated with 60, 120, and 180 µl/mL of LR038 was evaluated by using the fluorescent probe, H₂DCFDA. It was observed that at the two highest concentrations, there was a significant increase in the accumulation of the ROS when compared with the untreated cells ($P < 0.05$, $P < 0.01$) (Fig. 3). The accumulation of the ROS was approximately 2x and 3x at the concentrations of 120 µl/mL and 180 µl/mL, respectively.

3.5 Evaluation of the mitochondrial potential of the *Phytomonas serpens* promastigotes

The *P. serpens* promastigotes that were treated with 60, 120, and 180 µg/mL of LR038 were stained with Rhodamine 123 and evaluated by flow cytometry, to verify the effects of the lyophilized residue. The cells that were treated with 120 and 180 µl/ml of LR038 showed a reduction in the mitochondrial potential when compared with the untreated cells. The Rhodamine 123 total fluorescence histogram (Fig. 4C) and the index of the variation results (Fig. 4A) showed a reduction in the fluorescence at concentrations of 120µl/mL and 180µl/mL, respectively. These two concentrations reduced the fluorescence by 44.6% (IV= -0.446) and 46.8% (IV= -0.468), respectively. These reductions did not differ statistically from the reduction of fluorescence in the positive control, 56.0% (IV= -0.560). These results were confirmed by the statistical analysis of the median of fluorescence, which was obtained and compared with the negative control ($P < 0.05$) (Fig. 4B).

3.6 Qualitative chemical profile of the residue.

The qualitative chemical profile of LR038 was determined by analytical liquid chromatography (Fig. 5A). The presence of 17 peaks and their respective ultraviolet spectra were observed, which were used to identify the classes of the secondary metabolites that were present in LR038. The results of this qualitative analysis indicated the presence of phenolic acids and flavonoids (Fig. 5B).

Discussion

The global essential oils market had a turnover of 18.62 billion US dollars in 2020, and strong growth is estimated for the next few years, reaching a value of 33.26 billion US dollars by 2027, with estimated sales of 473 .31 thousand tons of essential oils (Grand View Research, 2020). Although there is a great demand for these products, the essential oil yield from many plant species is almost always low (Alighiri et al., 2017). In contrast, millions of tons of waste are generated during production and they are disposed of into the environment, in some cases, without treatment (Peshev, 2020). Although these residues are discarded, they have many biologically active molecules, which exhibit diverse biological activities. The residue from the hydrodistillation of *Dittrichia graveolens*, with a high content of flavonoids, presented antioxidant, antibacterial, and anti-inflammatory activities, with the advantage of not being cytotoxic to the fibroblasts (Gharred et al., 2021).

The genus *Phytomonas*, belonging to the Trypanosomatidae family, has pathogenic members, which are parasites that cause diseases in plants and humans. Some species of this genus cause severe infections in large crops, such as acute lethal wilt in oil palm and coconut trees (*Phytomonas staheli*), necrosis in coffee phloem (*Phytomonas leptovorum*), and root bubbling in cassava (*Phytomonas francai*) (Jaskowska et al., 2015). Despite not causing a severe systemic disease, *P. serpens* can compromise the commercial value of tomato plants, due to the appearance of yellow spots on the parasitized fruits (Schwelm et al., 2018). However, this species constitutes an important biological

model, as it presents natural characteristics similar to other pathogenic members belonging to the Trypanosomatidae family. In addition, unlike other pathogenic species, *P. serpens* is readily isolated and cultivated in the laboratory, enabling in vitro studies in the most diverse areas.

Studies have proven that compounds from medicinal plants have important molecules, with antimicrobial activities on the promastigote forms of *P. serpens*. *Piper crassinervium* and *P. amalago* leaf extracts have inhibited the *P. serpens* proliferation in vitro, with low to moderate cytotoxicity in mammalian cells (Cancini et al., 2020). Silva et al. (2019) reported that the use of the essential oil of *Varronia curassavica* inhibited the growth of *P. serpens*, causing a loss of the cell membrane integrity. Pereira et al. (2022) showed an inhibitory effect of *L. camara* essential oil on the promastigote forms of this same species. Thus, this study aimed to evaluate the effect of residual water from the hydrodistillation process of *L. camara* leaves, as a source of natural compounds for the control of protozoa of the genus *Phytomonas*.

In this work, it was possible to demonstrate the antiprotozoal actions of the residues from the production chain of essential oils from four accessions of *L. camara* on *P. serpens*. The results have shown that the four samples of the lyophilized residues evaluated showed antiprotozoal activities at low concentrations, with the IC₅₀ value ranging from 13.68 to 43.69 µg/mL. Although the four LRs showed a promising inhibition of parasite viability, LR038 was selected for having a lower IC₅₀ value.

From the qualitative chemical profile of the LR038 sample, two classes of natural products were detected, the phenolic acids and flavonoids, which can be attributed to the detected trypanocidal activities. Although the molecules from these classes have known antiprotozoal activities, there are no reports on the activity of these molecules against *P. serpens*. Caffeic acid, belonging to the phenolic acid class, is a good representative to demonstrate the antiprotozoal capacity of this group of compounds. Silva Bortoleti et al. (2019) reported that caffeic acid could inhibit the proliferation of the *Leishmania amazonensis* promastigotes, and cause changes in the cell morphology through the loss of plasma membrane integrity, as well as with the accumulation of the levels of the reactive oxygen species, thus increasing the oxidative response. Flavonoids also have antiprotozoal activities, like quercetin and myricetin, which have shown a trypanocidal activity on *Trypanosoma brucei*, the etymological agent of African human trypanosomiasis (Larit et al., 2021).

Since it was shown that LR038 markedly decreased the viability of *P. serpens* at low concentrations, the effects of this residue on the cell membrane integrity, on the intracellular levels of the reactive oxygen species, and on the mitochondrial membrane potential of the *P. serpens* promastigotes, were determined.

One of the possible mechanisms of action of natural products on cell viability includes their effects on the integrity of the plasma membrane. The evaluation that was based on the permeability of propidium iodide showed that the treatment with LR038 did not alter the integrity of the plasma membrane of the *P. serpens* promastigotes. This might be due to the low liposolubility of LR038, as molecules typically

require that the hydrophobic groups interact directly with the membrane (Jesus et al., 2021). The hydroxyl (polar) groups that were present in the flavonoids and phenolic acids likely entered the cell through controlled endocytosis.

The biological activities of flavonoids are mainly related to their antioxidant effects. However, several studies have also demonstrated the pro-oxidant action of many molecules of this class of natural products. A good example is quercetin, which is capable of inducing the reactive oxygen species in human embryonic stem cells (hESCs), thus triggering selective cell deaths (Kim et al., 2017). Another representative of the flavonoid class with an oxidizing action is cynaroside, which inhibits the growth of the *Leishmania donovani* promastigotes, by inducing the production of ROS (Tabrez et al., 2021).

The ROS levels in the *P. serpens* promastigotes that were treated with LR038 were determined by using H₂DCFDA, a non-fluorescent probe that when permeating the cell, is converted into dichlorodihydrofluorescein (DCFH) by the action of the esterases. In the presence of free radicals, such as ROS, DCFH is oxidized and converted to 2,7-dichlorofluorescein (DCF), a highly fluorescent product. The results obtained revealed that an intracellular increase in the ROS was accompanied by an increase in the concentration of LR038. Notably, the levels of ROS that were detected in the parasites that were treated with 120 and 180 µg/mL of the residues were approximately 2x and 3x higher than in the untreated parasites.

Reactive oxygen species result from cellular metabolism, and their levels are regulated by the presence of an endogenous antioxidant complex that when affected, can lead to an intracellular accumulation of ROS. The consequent oxidative stress generated might cause metabolic changes, including a loss of the mitochondrial membrane potential (Fonseca-Silva, et al., 2015, Mittler, 2017). In this study, reductions of 44.6% and 46.8% in the Rhodamine-generated fluorescence were observed in the *P. serpens* promastigotes that were treated with 120 and 180 µg/mL of LR038, respectively. This reduction in the fluorescence demonstrated a decrease in the mitochondrial membrane potential that was generated by LR038, which might be related to the increase in ROS that was observed in the cells that were treated with this residue.

Since trypanosomatids have a single mitochondrion, the proper functioning of this organelle is essential for the survival of these organisms, hence constituting an important target for the construction of drugs (Menna-Barreto and Castro, 2014, Fidalgo and Gille, 2011). A study by Bombaça et al. pointed out that the mitochondrial dysfunction and the production of ROS were the main determinants of the antiprotozoal activity of the naphthoimidazoles in *Trypanosoma cruzi* (Bombaça et al., 2019). Similarly, the apigenin flavonoid has been shown to exert antileishmanicidal activity by altering the membrane potential, as the result of an increase in the production of ROS (Fonseca-Silva et al., 2015).

When considering the results that were obtained here, the antiprotozoal activities that were presented by the residues of the hydrodistillation of the essential oil of *L. camara* were due to its ability to induce oxidative stress. This was evidenced by the increase in the ROS levels and the functional alterations in

the mitochondria, which interfered with the production of energy that compromised the parasite's survival.

Conclusions

The residues from the hydrodistillation of the *L. camara* leaves can serve as a source of biologically active compounds, phenolic acids, and flavonoids, with an antiprotozoal potential. The highest concentrations tested promoted changes in the potential of the mitochondrial membrane and caused the accumulation of the reactive oxygen species. This demonstrated the effectiveness of the residues that were generated during the hydrodistillation of *L. camara* leaves to obtain the essential oils.

Declarations

6.1. Ethical Approval

Not applicable.

6.2. Consent to Participate

Not applicable.

6.3. Consent to Publish

Not applicable.

6.4. Authors Contributions

Caroline Alves Soares: Conceptualization, Data curation, Methodology, Roles/Writing - original draft. **Tamírís Aparecida de Carvalho Santos:** Conceptualization, Methodology. **Luís Fernando de Andrade Nascimento:** Conceptualization, Methodology. **Raphael Amancio de Jesus:** Conceptualization, Methodology. **Arie Fitzgerald Blank:** Conceptualization; Writing - Review & Editing; Funding acquisition. **Ricardo Scher:** Conceptualization, Methodology. **Valéria Regina de Souza Moraes:** Conceptualization, Methodology. **Roberta Pereira Miranda Fernandes:** Conceptualization, Methodology, Writing - Review & Editing. **Maria de Fátima Arrigoni-Blank:** Conceptualization, Writing - Review & Editing; Supervision.

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6.6. Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6.3. Author contributions

Caroline Alves Soares: Conceptualization, Data curation, Methodology, Roles/Writing - original draft. **Tamíris Aparecida de Carvalho Santos:** Conceptualization, Methodology. **Luís Fernando de Andrade Nascimento:** Conceptualization, Methodology. **Raphael Amancio de Jesus:** Conceptualization, Methodology. **Arie Fitzgerald Blank:** Conceptualization; Writing - Review & Editing; Funding acquisition. **Ricardo Scher:** Conceptualization, Methodology. **Valéria Regina de Souza Moraes:** Conceptualization, Methodology. **Roberta Pereira Miranda Fernandes:** Conceptualization, Methodology, Writing - Review & Editing. **Maria de Fátima Arrigoni-Blank:** Conceptualization, Writing - Review & Editing; Supervision.

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Figures

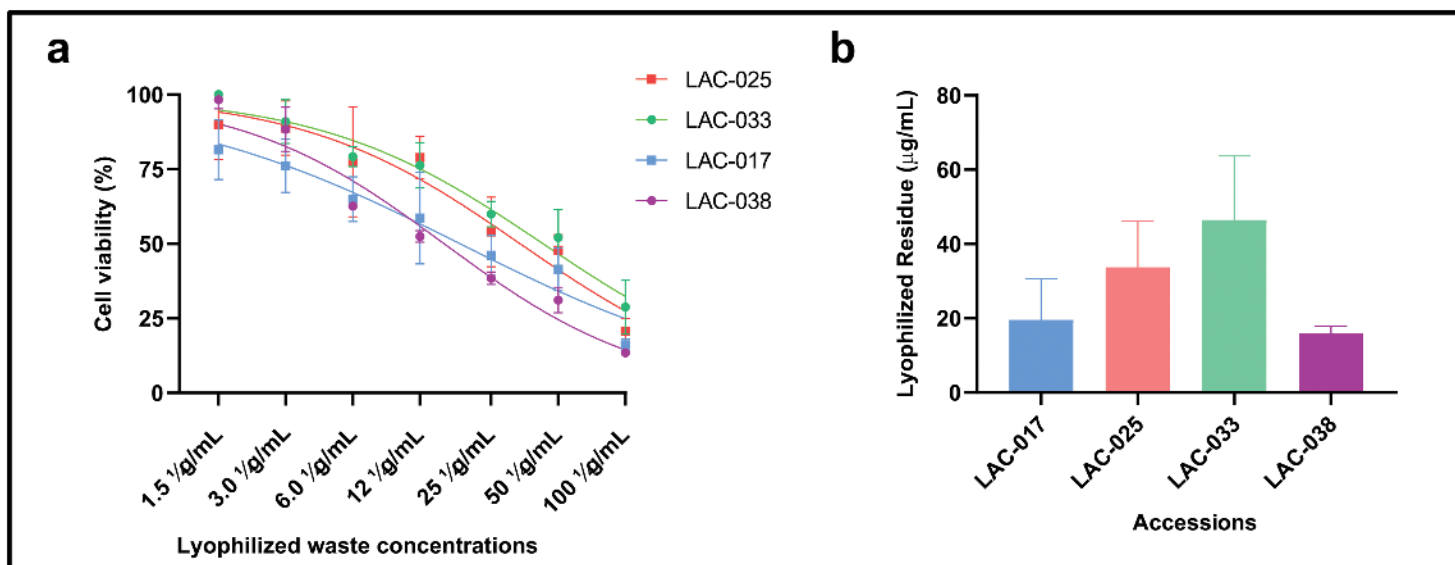


Figure 1

Effects of the lyophilized residue from four *Lantana camara* L. accessions on the promastigote forms of *Phytomonas serpens*. The parasites (1×10^5 cells/ml) were treated with 1.5; 3.0; 6.0; 12.0; 25.0; 50.0, and 100 $\mu\text{g/mL}$ for 48 hours, and the viability was evaluated by the resazurin colorimetric test. (a) Cell viability. (b) Values (IC_{50}) of the lyophilized residue. The data is expressed as the mean $\pm$ standard error of three independent experiments.

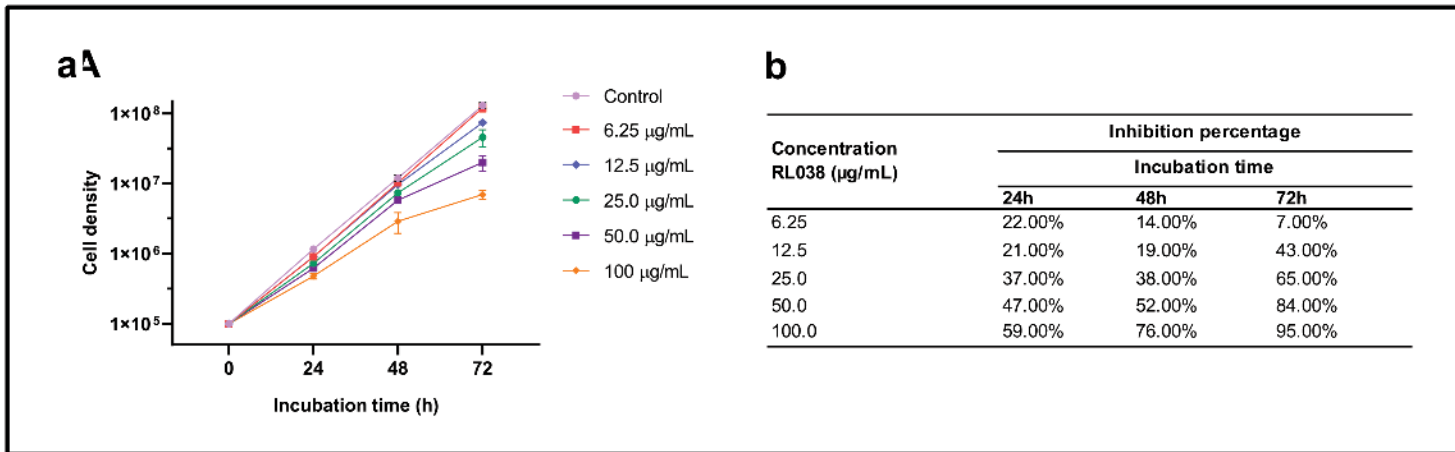


Figure 2

Effects of the lyophilized residue from the *Lantana camara* L. accession LAC-038, named LR038, on the growth of the *Phytomonas serpens* promastigotes. The parasites (1×10^5 cells/ml) were treated with 6.25; 12.5; 25.0; 50.0, and 100 $\mu\text{g/mL}$ of the lyophilized residue for 72 hours, with direct counting performed every 24 hours. (a) Cell growth curve. (b) Percentage of inhibition. Control equal to the untreated cells. The data is expressed as the mean $\pm$ standard error of three independent experiments.

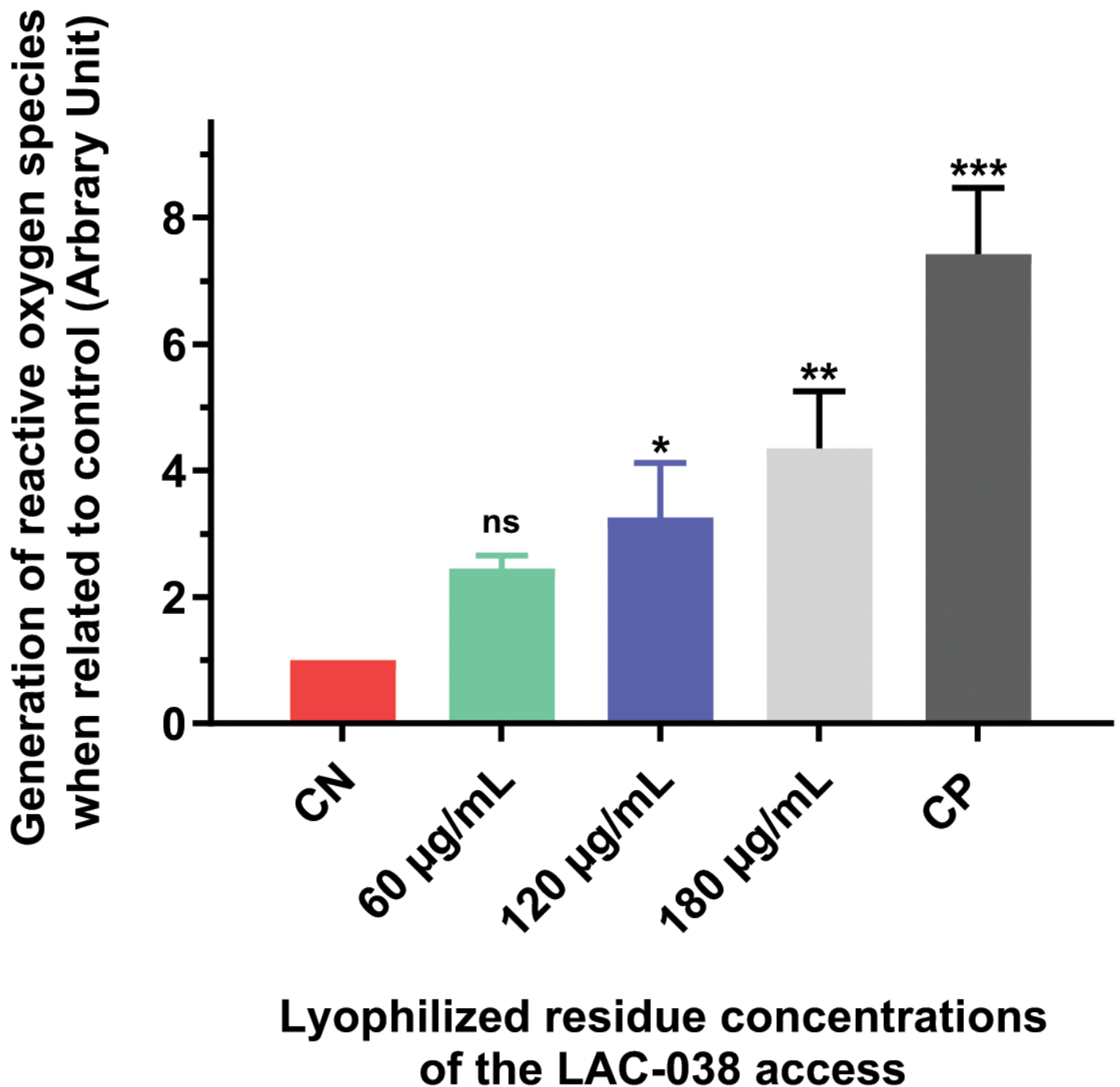


Figure 3

Effects of the lyophilized residue from the *Lantana camara* accession LAC-038, named LR038, on the production of the reactive oxygen species in the *Phytomonas serpens* promastigotes. The parasites (5,105 cells/ml) were treated with 60, 120, and 180µg/ml of the lyophilized residue for 48 hours. The EROS levels were assessed spectrofluorimetrically when using the H₂DCFDA probe. The untreated cells represent the negative control (CN), and the cells incubated with H₂O₂ (750 µM, 30 min) correspond to the positive control (CP). The fluorescence data was relativized with the negative control. The results

were expressed as the mean $\pm$ standard error of three independent experiments. The data was submitted to the Analysis of Variance (ANOVA) and the Tukey test, (*) $P<0.05$, (**) $P<0.01$, (***) $P<0.001$, while (ns) was not significant.

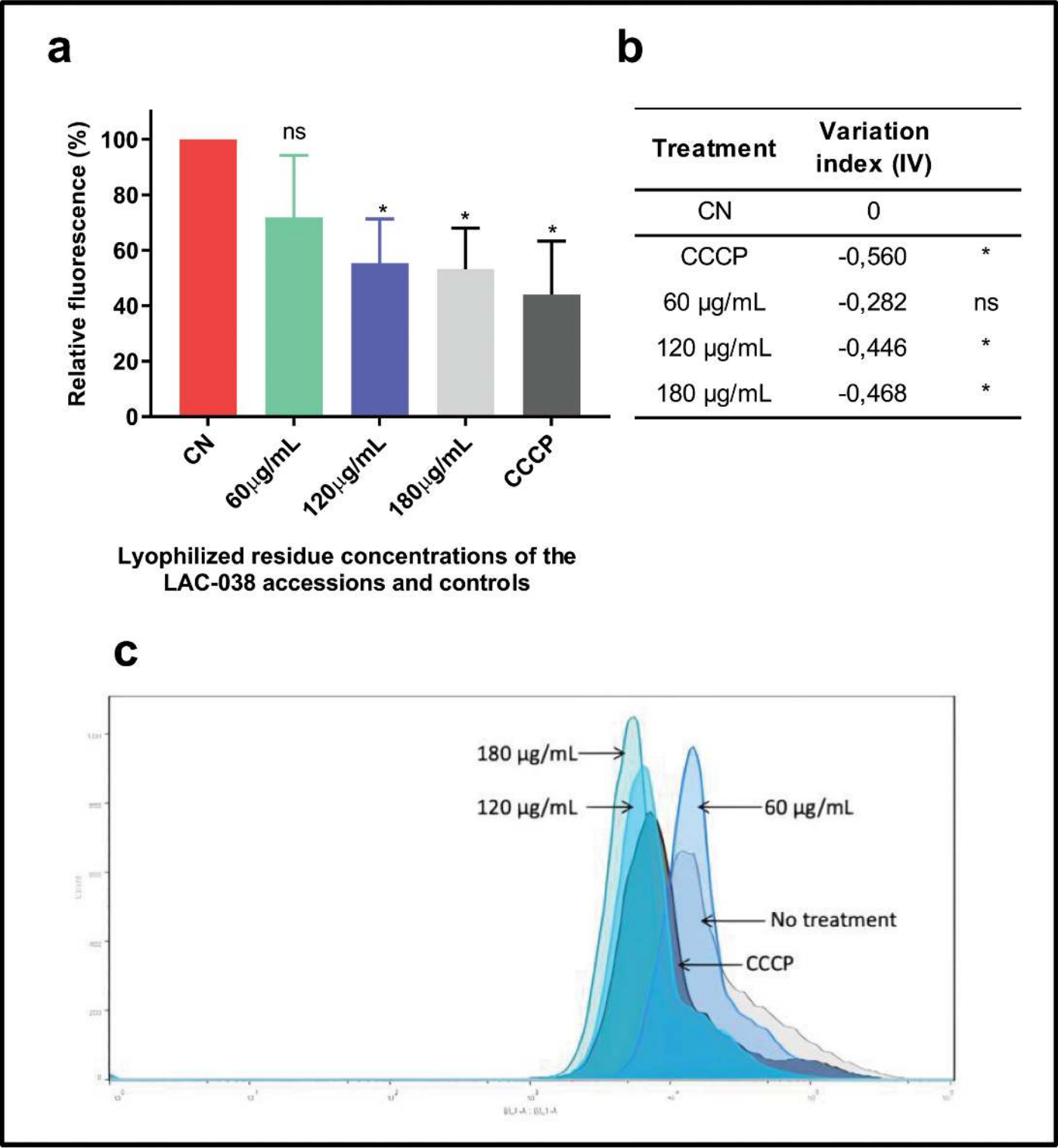


Figure 4

Effects of the lyophilized residue from the *Lantana camara* accession LAC-038, named LR038, on the mitochondrial potential of the *Phytomonas serpens* promastigotes. The parasites (5,105 cells/mL) were

treated with 60, 120, and 180µg/mL of the lyophilized residue for 48 hours and stained with Rhodamine 123, and evaluated by flow cytometry. The untreated cells represent the negative control (CN), and the cells incubated with CCCP correspond to the positive control (CP). (a) Representative histogram of the mitochondrial membrane potential. (b) The medians of fluorescence relative to the negative control. (c) The variation index results were expressed as a percentage of the relative fluorescence $\pm$ standard error. (*) Indicates the statistical difference to the untreated control ($P<0.05$), while (ns) was not significant.

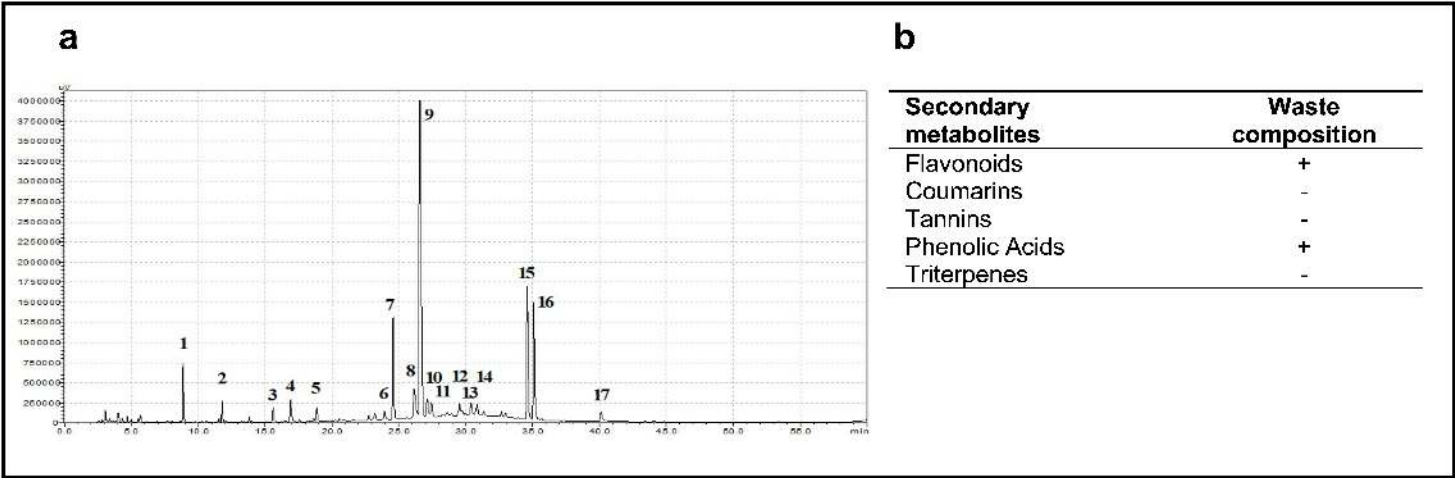


Figure 5

Screening of the *Lantana camara* L compound classes. (a) Chemical profile of the lyophilized residue from the accession LAC-038, named LR038. Phenolic Acids (Peak 1 - 6), Flavonoids (Peak 11 - 17), not clear (Peak 7 -10). (b) The classes of the compounds that were detected in the lyophilized residue of the accession LAC-038 (-) Absence (+) Presence.

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