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Research Article

Cytotoxicity Effects and Mechanisms of Action of *Cymbopogon Citratus* Essential Oils Against Pathogens

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Abstract

The essential oil of *Cymbopogon citratus* L. (DC) Stapf was found to be active against sensitive and multi-drug resistant strains of *Staphylococcus aureus*, Candida species, and protozoan strains including *Leishmania* species. The numerous reports on the anti-pathogenic activity of *C. citratus* warrant more research to elucidate the mechanism of action against the pathogens, as well as the cytotoxicity effects in human cells. The current study investigated the mode of action of the essential oil on *Leishmania* promastigotes, and its cytotoxicity effects on normal and cancer mammalian cells lines, using various colorimetric assays. Our results show that *C. citratus* triggers a programming death on *Leishmania* promastigotes inducing apoptosis. *C. citratus* also presents toxicity against HeLa and Vero cell lines and induces haemolyses.

Keywords: *Cymbopogon citratus; Cytotoxicity; Anti-Parasitic and Antibacterial Activity; Cytotoxicity; Haemolyses*

Abbreviations

C. citratus: Cymbopogon citratus; DMSO: Dimethyl sulfoxide; EO: Essential oil; Gram (+): Bacteria Gram positive *L. infatum: Leishmania infantum*; LDH: Lactato dehydrogenase; MTT - 3-[4, 5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide]; RBC – Red Blood Cells; *S. Aureus - Staphylococcus aureus; S. epidermidis: Staphylococcus epidermidis*

Introduction

Cymbopogon citratus (DC) Stapf., Poaceae, Family Gramineae, is commonly known as lemon grass due to its attractive scent reminiscent of lemon juice. It is a tall tropical plant from South Asia and Southeast Asia [1]. It is traditionally used in cooking and is often found in herbal supplements and teas.

Traditional doctors use aromatic plants since ancient time for their medical properties. Ethnopharmacological studies in several Angolan regions revealed that *C. citratus* is widely used in folk medicine to treat some skin infections, feverish and anti-inflammatory conditions, digestive disorders, diabetes, as well as other health problems. The use of *C. citratus* infusions to treat feverish and unwell conditions is widespread in the Angolan population. It has also been proven to have potent antibacterial, antifungal and anti-parasitic activity, as well as anxiolytic, hypnotic and anticonvulsant properties [2-6]. *Cymbopogon citratus* essential oils are also broadly used in food or drinks, as well as in perfumery, body care products and soap manufacture [7]. Since the last decade, particular attention has been given by researcher to the pharmacological properties of essential oils against several pathogens. Polyphenols and mono- and polymeric flavonoids, such as luteolin, quercetin, kaempferol, and apigenin glycosides and proanthocyanidins, were thought to be partially responsible

for its therapeutic potential [8-10]. Indeed, naturally occurring molecules of essential oils are more and more considered as valid additives to conventional antibiotherapies [11]. Despite this reported range of pharmacological activity [1], little is known about the mechanism of action of *C. citratus* against pathogens and its cytotoxic effects on host mammalian cells.

In a previous article, we reported that ultrastructural and apoptosis are involved death mechanisms of *Leishmania* parasites [3]. Although based on rational strategies, the actual cytotoxicity effect of the *C. citratus* essential oil remains to be elucidated. We, therefore, examined the different processes by which *C. citratus* essential oil induced cytotoxicity on mammalian cell line and evaluated it´s haemolytic effects.

Materials and Methods

Plant material and distillation of essential oil

Fresh leaves of *C. citratus* (DC) Stapf. were collected from the fields in Benguela - Angola. The plants were identified by Professor Pedro Catarino and voucher specimen were deposited at the herbarium of the Clinical and Industrial Laboratory of Benguela (ISPB).

Essential Oil

The plant leaves were dried at room temperature (26 ± 2˚C) for 5 days. The dried aerial parts were chopped into small pieces and subjected to hydro-distillation in a modified Clevenger–type apparatus for a minimum of 4 hours, according to the procedure described in the European Pharmacopoeia [12]. The obtained essential oils were collected and stored in hermetically sealed vials covered with aluminium foil to protect the contents from the light refrigerated at 4˚C.

Identification of essential oil components

Gas chromatography mass spectroscopy (GC/MS) techniques were performed to evaluate the composition of the essential oil. This technique was carried out in a Hewlett–Packard 6890 gas chromatograph fitted with an HP1 fused silica column, interfaced with a Hewlett–Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced Chem-Station software, version A.03.00. The retention indices from SPB-1 and SupelcoWax-10 columns as well as the mass spectra of the essential oil components were used to identify it´s elements. The retention indices were calculated by linear interpolation relative to retention times of C8-C23 of n-alkanes and compared with those of authentic standards included in our laboratory database.

Transmission and scanning electron microscopy

Leishmania infantum promastigotes were exposed to essential oil at concentrations that inhibit viability by 50% (IC_{50}) and cellular ultrastructural studies we performed using transmission and scanning electronic microscopy. Briefly, for the transmission microscopy cells were fixed with glutaraldehyde in sodium cacodylate buffer, post-fixing in osmium tetroxide and uranyl acetate. Cells were then dehydrated in ethanol and in propylene oxide and embedded in Epon 812 (TAAB 812 resin). Ultrathin sections were stained with lead citrate and uranyl acetate. For Scanning electronic microscopy, the samples were fixed and post fixed as described for transmission, dehydrated in ethanol, critical point dried using CO_2 and sputter-coat with gold. The specimens were examined in JEOL JEM-100 SX transmission electron microscopy (TEM) at 80 kV and in JEOL JSM-5400 scanning electron microscope (SEM) at 15 kV.

Cell cycle arrest

Parasites were incubated with essential oil at IC_{50} concentrations for 24 hours. After 24 h of incubation, cells were fixed with paraformaldehyde for 30 min at 4˚C and permeabilized by Triton X-100 for the intracellular staining of the DNA. Parasites were washed twice in PBS, span at 850g in a centrifuge, and treated with ribonuclease (50 µl of a 100 µg/ml stock of RNase) to ensure only DNA, not RNA, is stained. The cell cycle analysis was performed by flow cytometry after staining with 200 µl of Propidium iodide (stock solution 50 µg/ml).

Cell Toxicity assays

Sample preparation

The *C. citratus* essential oil was diluted in 100% DMSO4 to make up a stock solution of 200 µg/ml which was diluted to six 2-fold and six 10-fold concentration dilutions in complete medium to final concentration ranges of 1000 µg/ml - 31.5 µg/ml and 1000 µg/ml - 0.001 µg/ml respectively.

Measurement of cellular metabolic activity

MTT assay

Vero cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% of heat-inactivated fetal bovine serum, at 37°C in a 5% CO_2 gas controlled environment. For the experiments, cells were washed with Ca-PBS, detached with trypsin and counted using an automatic cell counter to determine the cell plating density.

Cell viability relative to untreated controls was measured using a colorimetric (3-[4, 5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) assay. Cells were seeded at a density of $1x10^5$ cells/ml and incubated with 2-fold and 10-fold serial dilutions of test samples and incubated for 48h. The plate was developed by $DMSO_4$ after 4hr incubation with MTT (5 mg/ml). The absorbance was read at 540 nm. IC_{50} (50% inhibition of cell viability) value was derived from dose-response curves. Emetine (2 mg/ml stock solution) was used as a positive control.

Resazurin assay

Cell viability was confirmed by alamar blue assay, using Vero and Hela cell lines.

Vero cells were seeded at a density of $1x10^4$ cells/ml and incubated with a range of concentrations of the test sample at 37°C in a 5% CO_2 incubator for 24hr. Following incubation, $1/10^{th}$ of alamar blue reagent was added to the cells. Cells were incubated for further 4hrs, protected from direct light. Absorbance was read at 570 nm, using 600 nm as a reference wavelength.

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin/amphotericin B. Cells were plated in 96-well plates at a density of $1x10^4$ cells per well and incubated overnight at 37°C in a 5% CO_2 incubator. Five-fold serial dilutions of the test extracts were prepared in medium and 100 µL added to the cells in duplicate wells per concentration to achieve a final concentration range of 100 – 0.16 µg/mL. After 24h incubation with the extracts, cell viability was assessed by removing the medium containing the extracts and replacing it with medium supplemented with 10% (v/v) resazurin reagent (*In vitro* toxicology assay kit, Sigma-Aldrich). After an additional 2h incubation at 37°C, fluorescence was measured in a BioTek Synergy MX plate reader using excitation/emission wavelengths of 560 nm and 590 nm. Background fluorescence readings were obtained from wells without cells and subtracted from all other readings. Cell viability in the wells exposed to extracts was calculated as a percentage of the fluorescence values obtained in wells containing cells and medium only. Percentage cell viability vs. log (extract concentration) was plotted using GraphPad Prism. Where appropriate (i.e. when a sigmoidal dose-response was observed) non-linear regression analysis was performed to obtain 50% inhibitory concentration (IC_{50}) values.

Cell Permeabilization – LDH assay

Cell membrane permeability was measured using Lactate Dehydrogenated (LDH) assay. Aliquots of 100 µl of cells were inoculated in 96-well plates at densities of $1x10^4$ cells/ml. Ranges of concentrations of the test sample were added, and the plate was incubated for 24h at 37°C in a CO_2 humidified incubator. Four controls were included in each experiment: background control (medium only), cell without treatment to measure the spontaneous release of LDH by cells, maximum LDH release (high control) were Triton x-100 was used

to disrupt the cytoplasmic membrane aim the maximum value LDH release and the test substance control, to measure interference of the essential oil. After 24h, the culture plate was centrifuged at 400g for 5 min and 100 µl of the supernatant was carefully transferred to corresponding wells of an empty 96 well flat bottom plate. The reaction mixture containing LDH substrate was added, and the plate was left for 30 min to develop at room temperature, in the dark. Absorbance was read at 490 nm. The reference wavelength was set at 680 nm. Cytotoxicity (%) was calculate as: (experiment value – background control /Maximum LDH release - background control)x100.

Sulforhodamine B assay

The cell anti-proliferation effect was measured using Sulforhodamine B (SRB) assay. Cells were plated at different densities from $1x10^3$ to $1x10^6$ to determine density that gives optimal OD at 100% growth, and to ensure cells were within the exponential growth phase for the duration of the experiment. Cells were inoculated into two 96-well plates at densities of $1x10^4$ and $1x10^5$ cells/ml and incubated for 24hrs. After 24hrs one plate was fixed with 50% trichloroacetic acid (TCA) to represent a measurement of the population at the time of drug addition (T = 0). The other inoculated plates were treated with the test samples at different concentration ranges and then incubated for 48hrs. Cells without drug served as control. The blank contains complete medium without cells. The plates were fixed with cold TCA (50%) for 2hr at 4°C, then washed under slow running tap water and air dried. Plates were stained with SRB (0.4%) for 30min, then washed 5 times with acetic acid (1%) and air dried. Protein-bound dye was extracted with 10 mM Tris base (pH10.5) for optical density determination at the wavelength 510 nm.

The optical density of the test well after 48-h period of exposure to test samples is T, the optical density at time zero is T0, and the control optical density is C. Four response parameters, Growth inhibition of 50% (GI_{50}) where 100 x (T-T0)/(C-T0) = 50), Total Growth Inhibition (TGI) concentration calculated from T = T0, 50% lethal concentration (LC_{50}) was calculated from [(T-T0)/T] X100, and LC_{100} (100% lethal concentration) was determined.

Haemolytic assay

During the antimalarial studies, there was evidence of red blood cell lysis. A haemolysis assay was performed in order to determine the erythrocytes lysis extent. The effect of essential oil on human red blood cells (RBC) was evaluated through haemolytic assay using Red Blood Cells (RBC) isolated from fresh blood. RBC were separated from serum by centrifuging whole blood at 1000g for 10 minutes. Isolated RBC were washed with 0.9% NaCl (1:9 v/v). The pellet was diluted with NaCl (1:24 v/v), and aliquots of 100 µl were exposed to a range of concentration of the test sample followed by incubation for 30 min, 1 hour, and 3 hours. The presence of haemoglobin was assessed at these intervals by reading absorbance at 540 nm using a plate reader.

Results and Discussion

Essential oil analyses

The essential oil analysis revealed that Geranial (40,6%), Neral (28,3%) and Myrcene (10,5%) were the main compounds of *C. citratus* essential oil. Citral, a combination compound of the bioactive isomers Geranial and Neral accounts for 68,9% of total compounds (Table 1). Other major constituents included Monoterpene hydrocarbons (12.3%), N-Myrcene (11.5%), and Geraniol (1.3%).

The volatile fraction of *C. citratus* essential oil revealed the presence of 10 different compounds accounting for 83.86% of total peak area. The trace elements were not shown in Table 1.

Components	RI a)	Peak area (%)
6-methyl-5-hepten-2-one	8.725	0.97
Myrcene	8.875	10.5
α-(Z)-Ocymene	10.533	0.22
β-(E)-Ocymene	10.917	0.27
Linalool	12.883	0.5
Citronelal	14.25	0.11
β-citral (Neral)	18.025	28.26
Geraniol	18.458	2.37
α-citral (Geranial)	19.142	40.55
Linalool isobutyrate	22.967	0.11

Table 1: *Major compounds of C. citratus essential oil determined by GC-MS.*
a) Retention index relative to n-alkanes

Mechanism of action

Ultrastructural studies

To further understand the bioactivity mechanisms of *C. citratus* essential oil, we performed several mechanistic studies, namely ultrastructural studies, depolarization of the membrane potential and cell cycle arrestment. These studies were performed using *L. infantum* promastigotes exposed to IC_{50} concentrations of *C. citratus* essential oil.

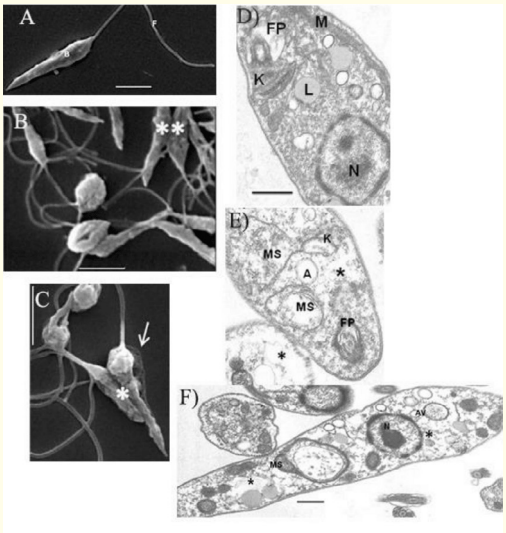


Figure 1: *Morphological alterations L. infantum promastigotes when exposed to essential oil at concentrations that inhibit viability by 50% (IC50). Scanning electron micrographs photographs (Figure A, B, and C) and transmission electron micrographs (Figure D, E, and F). Untreated cells showing the typical body form (B) with flagella (F) (Figure 1A and 1E). Observe the irregular surface (asterisks) and membrane disruption (arrows) in Figure 1B and 1C. Note the mitochondrial swelling (MS) Figure 1E alterations on the cytoplasm organization, nuclous chromatin, and kinetoplast sweeling. (Figure 1E). Figure 1 D, E and F N. Nucleus, K. Kinetoplast, F. Flagellum, FP. Flagellar pocket, MB Multilamelar bodies, bars 2µm.*

Figure 1 illustrate the morphological changes observed on *L. infantum* promastigotes after incubation for 7h in the presence or absence of *C. citratus* essential oil. The images were obtained using scanning and transmission electron microscopy.

The untreated promastigotes show the general shape under scanning electron microscopy presenting an anterior flagella (F) and a typical elongated body shape (B) (Figure 1A). The transmission electron microscopy micrographs show that the parasites present normal flagellar pocket, mitochondria kinetoplast, and nucleus. After treatment with the essential oil, the parasites showed septation bodies with abnormal forms. The surface of the promastigotes presents blebs formation and leakage of intracellular content due to disruption of the membrane.

The same aberrant forms (Figure 1E) were also observed by transmission electron microscopy, where it´s possible to observe cytoplasmatic disorganization, cytoplasmatic clearing (Figure 1F) with an increase in autophagosomal structures (Figure 1E) it was also possible to observe swelling of the cell body (Figure 1E), mitochondria (Figure 1F) and kinetoplast (Figure 1E). The parasites nuclear chromatin shapes are similar to an apoptotic cell (Figure 1F).

Cell-cycle arrestment assay

Distribution of cell DNA through Cell cycle of *L. infantum* using Propidium Iodide staining was analysed by flow cytometry (Figure 2), after the parasites were exposed to IC_{50} concentrations of *C. citratus* essential oil. Following 24 h of incubation, the majority of treated parasite cells were arrested in G0/G1 phase of cell cycle (essential oil, 84%), opposite to what occurs in untreated cells (35.9%).

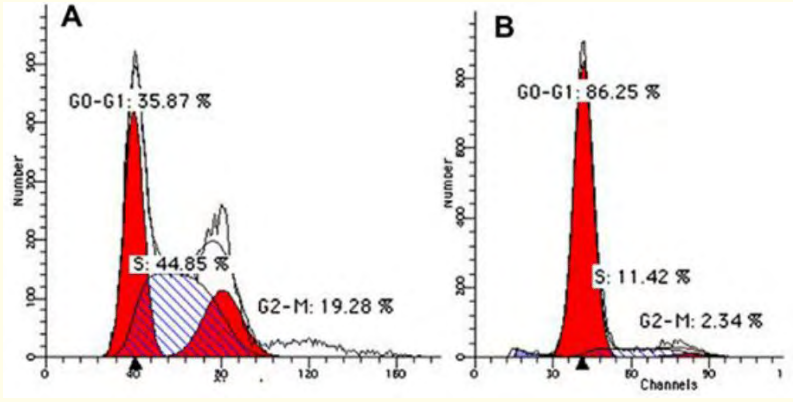


Figure 2: *Distribution of cell DNA trough cell cycle of parasites in the absence and presence of the C. Citratus essential oil.*

Cytotoxicity of *C. Citratus* Essential Oil

In order to evaluate the cytotoxicity effects of *C. citratus*, we performed several studies aiming the evaluation of membrane integrity and cell metabolic status as well as the haemolytic activity of the *C. citratus* essential oil. The membrane integrity of Vero cell was evaluated by the LDH assay, and the metabolic status was evaluated in Vero cells and HeLa cells using the MTT assay and the resazurin assay, respectively.

Haemolysis effect of *C. citratus* essential oil

C. citratus essential oil exhibit more that 50% haemolysis of human red blood cells (RBC) after 1 hour incubation and for concentrations of 100 µg/ml and 200 µg/ml. Low concentration of *C. citratus* essential oil presents no haemolytic effect (12 µg/ml) (Table 2).

	Concentration of *C. citratus* essential oil (µg/ml)				
Time Interval (Hours)	**200 µg/ml**	**100 µg/ml**	**50 µg/ml**	**25 µg/ml**	**12 µg/ml**
0.5	0.7	0.65	0.4	0.1	0
1	0.85	0.77	0.45	0.14	0
3	0.86	0.75	0.55	0.4	0.1

Table 2: *Haemolytic effect of C. citratus essential oil observed at 3 intervals; 30 min, 1 hour and 3 hours post exposure.*

LDH assay

The plasma membrane integrity of the mammalian cells, after treatment with *C. citratus* essential oil, was determined by the quantification of the plasmatic LDH activity present in the culture medium. Following incubation of Vero cell with medium containing *C. citratus* essential oil we could observe an increase in LDH release. This increase is concentration depended, increasing with the increase in *C. citratus* essential oil concentration.

In our experiments the untreated cells spontaneously released 15% of the maximum LDH activity. Concentrations of 10 µg/ml and below showed activity comparable to the negative control (Figure 3).

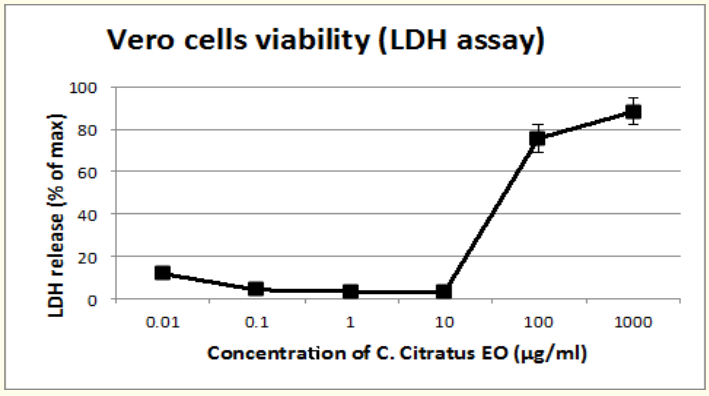
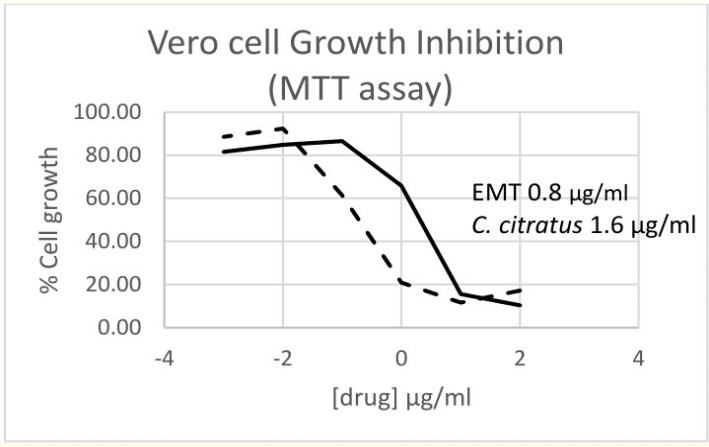


Figure 3: *The C. citratus essential oil effect on LDH release from Vero cells, after incubated for 24h at 37°C. Cytotoxicity percentage (%) was calculate as: (experiment value – background control /Maximum LDH release - background control) x100, being the maximum LDH released obtained by treatment of Vero cells with Triton X-100.*

MTT and resazurin assay

Cell viability as a function of redox potential was measured using the MTT and resazurin assay. The MTT assay was performed using Vero cells while the resazurin assay was performed using HeLa cells.



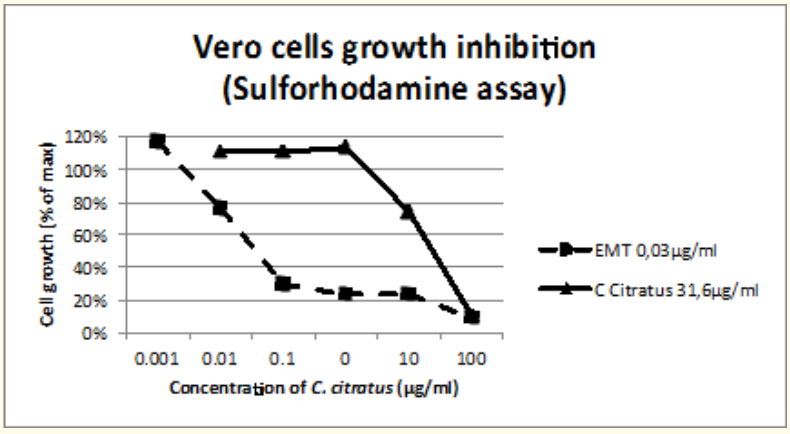


Figure 4: *The growth inhibitory effects of essential oil of C.citratus and negative control drug, emetine on Vero Cells. A) MTT assay B) Sulforhodamine B assay.*

Figure 4 shows the growth inhibition of Vero cells after treatment with *C. citratus* essential oil. It´s possible to observe that in the MTT assay the IC_{50} values of 0.15 µg/ml and 0.08 µg/ml for *C. citratus* and negative control drug, emetine, respectively. However, in the sulforhodamine assay, the values changed to GI_{50} = 31.6 µg/ml and 0.03 µg/ml for *C. citratus* and negative control drug, emetine, respectively.

An additional assay, was performed using Hela cells. In this assay the Hela cells were incubated with *C. citratus* essential oil, and cell viability was measured using the resazurin assay. Figure 5 shows the correlation between cell viability and concentration of *C. citratus*. It´s possible to observe that an increase in *C. citratus* concentration induces a reduction of cell viability with and IC_{50} of 0.95 µg/ml.

Despite of the last year's advances, in the knowledge of the bioactivity of the *C. citratus* essential oil, little is known about the mechanisms involved in the action of that essential oil.

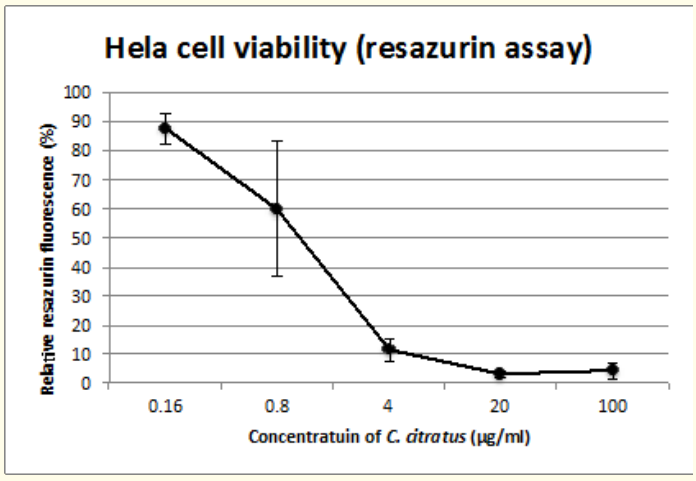


Figure 5: *Hela cell viability after 24h incubation with C. citratus essential oil. Cell viability was assessed by measuring the fluorescence (excitation/emission wavelengths of 560 nm and 590 nm) produced after incubation with resazurin reagent. Results express the average of 3 different experiment ± standard deviation.*

In recent years, several screenings of *C. citratus* essential oil have been carried [11,13,14], namely by our group [2-4], confirming the importance of *C. citratus* essential oil as source of novel molecules with several pharmacological properties. A composition analyses showed that citral, a combination compound of the bioactive isomeric aldehydes Geranial (trans-citral) and Neral (Cis-citral) accounts for 68,9% and myrcene that represents 10.5% of total compounds of the essential oil.

Twenty-three compounds were identified in *C. citratus* essential oil sample from Angola, representing 95% of the detected compounds. Table 1 represents the major 10 compounds that account for 83,8% of the total compounds. We also identified traces of Citronellyl acetate, E-α-Bergamotene, 2-Tridecanone, small amounts (0,1% of the total) of Perillene, Photocitral B, Photocitral A, Citronellal, Rosefurane epoxide and 2.3-Epoxyneral, 2.3-Epoxygeranial, and 0,2% of Z-Epoxyocimene Linalool and Photocitral A (data not shown). Previous reports on *C. citratus* essential oil presents similar results [3,7]. However, the Cuban essential oil presents different composition, with geraniol (52,3%), cis-pinocarveol (20,2), neural (9,8%) [15].

Leishmania promastigotes show an increase in cell and organelle volume and cytoplasm vacuolization after treatment with essential oil. In this sense, the essential oil may have a passive entry and may accumulate in parasite cell membranes leading to an increase of membrane permeability, as observed by propidium iodide assay. Similar ultrastructural alterations were also observed in Trypanosoma cruzi and *Leishmania amazonensis* treated with other essential oils and/or their main constituents [16].

The presence of autophagosomal structures was very prominent. Other authors also observed this alteration in *T. cruzi* and in *L. amazonensis* treated with drugs like ketoconazole, terbinafine, among others [17,18]. Another important alteration is the presence of membrane elaborated structures in the cytoplasm, appearing as myelin-like figures. The presence of these structures suggests the occurrence of an autophagic process, with the formation of structures known as autophagosomes [18]. These structures are probably involved in the breakdown and recycling of abnormal membrane structures, suggesting an intense process of remodelling of intracellular organelles irreversibly damaged by the essential oil.

The mitochondria, of the cells treated with the essential oil, revealed as less electron-dense, and are typically swelled. It is also possible to observe complex and elaborate structures with the inner mitochondrial membrane folded toward the mitochondrial matrix. These alterations have also been seeing in previous studies on *L. brasiliensis* [19] and *L. amazonensis* [20].

The main reasons for which a number of plant metabolites with bioactivity activity have not made into clinical evaluation is their high toxicity on mammalian cells. This lack of selectivity was observed on *C. citratus* essential oil, once they showed toxicity against mammalian cells tested, and can represent an obstacle to the future use in humans. The hydrophobic nature is a common feature of plants essential oils, which pointed the mechanistic and cytotoxic studies to the cell membranes as the primary target [21]. Our cytotoxic results show that *C. citratus* essential oil is toxic to the mammalian cell used in our study. The cytotoxic effect is mediated by permeabilization of the cytoplasmatic membrane and reduction of the redox potential of the cells, suggesting a decrease of the mitochondria activity. At concentration, lower than 10 µg/ml did not induced it was release of LDH on Vero cells. However, when the resazurin is used as an indicator of the redox potential of the cells, it´s possible to observe a decrease in Hela cells viability at a concentration of 0.16 µg/ml (IC_{50} = 0.95 µg/ml) of essential oil and IC_{50} = 1.6 µg/ml on Vero cells. Sulforhodamine assay revealed toxic effect with lower concentrations of essential oil (GI_{50} = 31 µg/ml).

In conclusion, the essential oil seems to exert activity through cellular and nuclear membrane disruption as observed on electron micrographs of *L. infantum* promastigotes exposed to 30 µg/ml of the essential oil. The cellular disruption was confirmed by LDH assay, which showed diffusion of the LDH enzyme as a result of increased membrane permeability or possibly membrane disruption at high concentrations of 100 - 250 µg/ml. Haemolysis of red blood cells at drug concentrations of 200 - 100 µg/ml further confirmed this observation. Swelling of the mitochondria evident on the micrographs suggest that the overall functioning of the mitochondria cold have been affected. This effect was confirmed using MTT and resazurin assay, which measures cell viability based on the functioning of the mitochondria. The cell growth inhibitory effect through cell arrest at G0/G1 phase of cell cycle effect of the oil as demonstrated through DNA distribution was evident from the sulforhodamine assay where the oil showed a cytostatic effect at a higher GI_{50} of 31 µg/ml. The paper has demonstrated through microscopy and enzymatic assays the mechanisms of action of essential oils of *C. citratus* against pathogens and host cells [22-29].

Conclusion

Taken together, results demonstrated that the pathogenic effects of *C. citratus* on *Leishmania* promastigotes can be attributed to apoptosis with arrestment on phase G0/G1 cell cycle phase and nuclear disorganization, with chromatin condensation. This process induces disruption of the cellular and nuclear membrane and changes in the promastigotes shape. The toxic effect can be explained by the reduction in the redox potential of the cells as well as by cellular membrane permeabilization. The use of these compounds in human formulations requires further investigation since our results showed a toxic effect at low concentrations of *C. citratus* essential oil.

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Conflict of Interest

We declare that there is no financial interest or any conflict of interest.

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