

EVALUATION OF INHIBITORY AND ANTIOXIDANT ACTIVITIES OF FREE ESSENTIAL OILS AND NANOEMULSIONS ON TRICHOMONAS VAGINALIS¹

AVALIAÇÃO DA ATIVIDADE INIBITÓRIA E ANTIOXIDANTE DE ÓLEOS ESSENCIAIS LIVRES E NANOEMULSÕES SOBRE TRICHOMONAS VAGINALIS¹

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ABSTRACT

Trichomoniasis, an infection caused by *Trichomonas vaginalis*, is the most common non-viral sexually transmitted infection worldwide. Due to Metronidazole's (MTZ) resistance to standard drugs, this study aimed to evaluate the efficacy of free and nanostructured essential oils (EO) as *Cymbopogon flexuosus* (lemongrass), *Eucalyptus globulus* (eucalyptus) and *Ocimum basilicum* (basil), besides antioxidant activities against *T. vaginalis*. The free and nanostructured EOs were tested for in vitro activity against *Trichomonas vaginalis*; trophozoites were treated with different concentrations (ranging from 0.25% to 1.5%) in order to establish the MIC and IC50 values. Oxidative stress was also evaluated by measuring lipid peroxidation by the detection of thiobarbituric acid reactive substances (TBARS). The MIC observed for lemongrass was 1.5% with free oil and 1.0% with nanoemulsion, while for basil it was 1.5% with free and nanostructured oils. Significant antiparasitic activities were observed in 12 and 24 hours exposure, respectively, inhibiting 100% of trophozoites. Antioxidant activity was also observed in these oils, which, either in free or nanostructured form, were able to reduce lipid peroxidation.

Keywords: *Cymbopogon flexuosus*, *Eucalyptus globulus*, Nanotechnology, *Ocimum basilicum*, Trichomoniasis.

RESUMO

Tricomoniase, infecção causada pelo Trichomonas vaginalis é a infecção sexualmente transmissível não viral mais comum do mundo. Devido a resistência do Metronidazol (MTZ), droga padrão, este estudo teve como objetivo avaliar a eficácia dos óleos essenciais (OEs) livres e nanoestruturados de Cymbopogon flexuosus (capim-limão), Eucalyptus globulus (eucalipto) e Ocimum basilicum (manjeriço) além de atividade antioxidante contra T. vaginalis. OEs livres e nanoestruturados tiveram a sua atividade in vitro

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testada contra *T. vaginalis*; trofozoítos foram tratados com concentrações diferentes (variando de 0.25% a 1.5%) a fim de estabelecer o valor de MIC e IC₅₀. O estresse oxidativo também foi avaliado medindo a peroxidação lipídica através da detecção de Substâncias Reativas ao Ácido Tiobarbitúrico (TBARS). O MIC observado para o capim-limão foi de 1.5% para o óleo livre e de 1.0% para nanoemulsão, enquanto o manjeriço foi de 1.5% para o óleo nanoestruturado e livre. Foi observado significativa atividade antiparasitária em 12 e 24 horas de exposição, respectivamente, inibindo 100% dos trofozoítos. Atividade antioxidante também foi observado nestes óleos, tanto na forma livre ou nanoestruturado foi capaz de reduzir a peroxidação lipídica.

Palavras-chave: *Cymbopogon flexuosus*, *Eucalyptus globulus*, Nanotecnologia, *Ocimum basilicum*, Tricomoníase.

INTRODUCTION

Trichomoniasis is the most common non-viral sexually transmitted infection in the world. This infection is caused by the flagellated protozoan *Trichomonas vaginalis* (MUZNY, 2018). This parasite is considered the main agent responsible for the occurrence of vaginitis in women, and has also been associated to infertility, cervical cancer, pelvic inflammatory disease, preterm birth, and low birth weight. Besides, it has been indicated that it is a risk factor for human immunodeficiency virus acquisition and transmission (KISSINGER, 2015).

Since 1959, metronidazole (MTZ) has been the drug of choice for the treatment of trichomoniasis. A 2 g single oral dose or 500 mg twice daily for 7 days is the recommended treatment. The latter regimen is suggested for HIV-infected or recidives patients. Side effects such as nausea vomiting and epigastric pain represent a barrier for patients finishing the protocol of treatment, which cause the emergence of resistant strains (DUNNE *et al.*, 2003; CUDMORE *et al.*, 2004).

Despite the known efficacy of MTZ, drug resistance has increased since 1962. Faced with several reports of resistance, natural products have been important sources of novel therapeutic options. The literature demonstrates the remarkable performance of essential oils (EOs), derived from the secondary metabolism of plants, showing the many biological activities of these compounds (BILIA *et al.*, 2014). Due to their lipophilic and chemical characteristics, EOs can be incorporated into nanostructures. Nanotechnology is an alternative to facilitate and improve their absorption and distribution to target cells, besides reducing volatility and instability through their incorporation into nanocapsules (COUVREUR; VAUTHIER, 2006; DANIELLI *et al.*, 2013; SALVIA-TRUJILLO *et al.*, 2015; CHANG *et al.*, 2015). Thus, the aim of this study was to evaluate the efficacy of essential oils of *Cymbopogon flexuosus* (lemongrass), *Eucalyptus globulus* (eucalyptus) and *Ocimum basilicum* (basil) against *T. vaginalis*, both free and nanostructured forms.

MATERIAL AND METHODS

Trichomonas vaginalis recovered and Method of culture

Samples were collected at the Clinic of Gynecology and Obstetrics in the Infant and Maternal Department of the School of Medicine (FAMED) of the Universidade Federal de Pelotas (UFPel), during routine medical appointments. Swab was to isolate and keep the axenic culture, trophozoites were inoculated in TYM (trypticase-yeast-extract-maltose) medium, pH 6.0, supplemented with 10% inactivated bovine serum at 37 °C (DIAMOND, 1957). Isolates were sub-cultured every 24 hours in new culture tube containing complete TYM medium and maintained at laboratory.

Ethics

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Ethics Committee of the School of Medicine, Federal University of Pelotas (Committee Approval number 873.180) and National Commission on Ethics in Research (amendment 1.838.829).

In vitro assay

The culture samples were collected and viable trophozoites were counted in a Neubauer cell-counter chamber, in triplicate, to adjusted the final density to 2.6×10^5 trophozoites/ml culture. The tests were performed at 37 °C at atmosphere of 5% CO₂, in nonoplicate, in 96 wells microtiter plates following SENA-LOPES *et al.*, (2018), adapted. From the stock solution with free and nanoemulsion oils (100%), dilutions in TYM medium were prepared at double the concentration desired for testing. Free and nanostructured oils were added to each well containing 100 µL of medium with *T. vaginalis* trophozoites to reach a final concentration of 0.25%, 0.5%, 1.0%, and 1.5%. The viability of the trophozoites in the culture plate was evaluated by 0.4% trypan blue dye exclusion and quantified in a cell-counter chamber using a hemocytometer. Also, the analysis determined the IC₅₀ (half the maximum inhibitory concentration) and evaluated the minimal inhibitory concentration (MIC) of the free oils or nanoemulsions that were able to inactivate 100% of viable trophozoites at the screening. To determine the MIC, the kinetic growth of cells was checked after 1, 6, 12, 24, 48, 72, and 96 h. MIC was confirmed by the failure of non-motile parasites to grow. Additionally, there was also the evaluation of four controls containing trophozoites with: TYM medium (negative control-NC), 25 µg/mL metronidazole Sigma-Aldrich® (positive control), control of Tween (TW) 80 0.01% (solubilizer) and blank nanoemulsions (BN). The same screening procedure was applied for all tests.

Essential oil

The essential oils of lemongrass (*C. flexuosus*) and eucalyptus (*Eucalyptus globulus*) were commercially obtained respectively from FERQUIMA Indústria e Comércio de Oliveira, and basil (*O. basilicum*) from Sigma Aldrich (São Paulo, Brazil) Trade Ltda.

Characterization of the essential oils

The characterization of free essential oils of lemongrass, eucalyptus and basil were performed at the Nanotechnology Laboratory of the Franciscana University of Santa Maria-RS (UFN), following the method by Hussain *et al.* (2008), adapted (GODOI *et al.*, 2017; DA SILVA GÜNDEL *et al.*, 2018a, 2018b).

Development of nanoemulsions

Nanoemulsions were developed by the Nanotechnology Laboratory (UFN) under high agitation method with homogenizator to incorporate the lemongrass, eucalyptus or basil EOs. The blank nanoemulsion was developed using a medium chain triglyceride, derived from caprylic and capric acids (GODOI *et al.*, 2017; DA SILVA GÜNDEL *et al.*, 2018a, 2018b).

Characterization of nanoemulsions

The physicochemical characterization of the formulations was evaluated at the same laboratory by determining the mean droplet size, polydispersity index, zeta potential and pH at the same day of production (DA SILVA GÜNDEL *et al.*, 2018a).

Thiobarbituric acid reactive species (TBARS) assay

This assay evaluates the levels of lipid peroxidation. It was performed as described by Ohkawa *et al.* (1979), modified. An aliquot of the sample (200 µl) was incubated with 8.1% sodium dodecyl sulfate (SDS) (200 µl), 0.8% thiobarbituric acid (TBA) (500 µl) and acetic acid/HCl (pH 3.4) (500 µl) at 95 °C during 60 min. The levels of TBARS were measured spectrophotometrically at 532 nm. The results were expressed as nmol TBARS/g tissue.

Statistical analysis

The results of the *in vitro* tests are presented as percentages and were analyzed by an analysis of variance (ANOVA) followed by comparison with the Tukey test using the Graph Pad Prism 7.04 software. The significance level was $p < 0.05$.

RESULTS

The nanoemulsion formulation used in this study has nanometric size, with the average size of all nanoemulsions ranging from 66 to 127 nm, a homogeneous size distribution as can be observed on the polydispersity index. The zeta potential was also measured like pH. The nanoemulsions containing *C. flexuosus*, *O. basilicum* and *E. globulus* had, respectively, average particle size of approximately 98 ± 0.82 , 113 ± 0.78 , 66 ± 0.81 ; polydispersion index of 0.20 ± 0.007 , 0.19 ± 0.007 , 0.23 ± 0.004 ; zeta potential of -7.48 ± 1.02 , -8.47 ± 0.98 , -7.53 ± 0.72 and acidic pH 4.00 ± 0.07 , 5.37 ± 0.04 , 5.90 ± 0.04 . The blank nanoemulsion maintained average particle size of approximately 127 ± 1.02 , polydispersion index of 0.25 ± 0.005 , zeta potential of -6.37 ± 0.58 , and pH of 5.15 ± 0.03 . The nanoemulsions possess adequate properties and morphology and preserved within 90 days the same quantity of major compounds when stored under refrigeration.

The major compounds were determined by gas chromatography. The major constituents identified in the *C. flexuosus* essential oil composition used in our study were β -geranial (45.74%), Z-citral (34.42%) and geraniol (6.01%). The components of *O. basilicum* oil also were determined by gas chromatography. The major constituents of this oil were estragole (87%), 1,8-cineole (3%) and trans-alpha-bergamotene (2%). The main components identified in the *E. globulus* oil were 1,8-cineole (75.7%); p-cymene (7.5%); alpha-pinene (7.3%); and limonene (6.4%).

The efficacy obtained with the free and nanostructured oils, MTZ and controls used for the *in vitro* assays can be observed at Figure 1. The inhibition of *T. vaginalis* trophozoites growth was dose-time-dependent, as are shown in the graphic (Figure 2).

Figure 1 - Antiparasitic activity of free (EO) and nanostructured (NE) essential oils of *Cymbopogon flexuosus*, *Ocimum basilicum* and *Eucalyptus globulus* against *Trichomonas vaginalis* after 24 hours of exposure. The *** indicate statistical differences when comparing the groups to the MTZ control by one-way ANOVA followed by Tukey post hoc tests ($p < 0.05$).

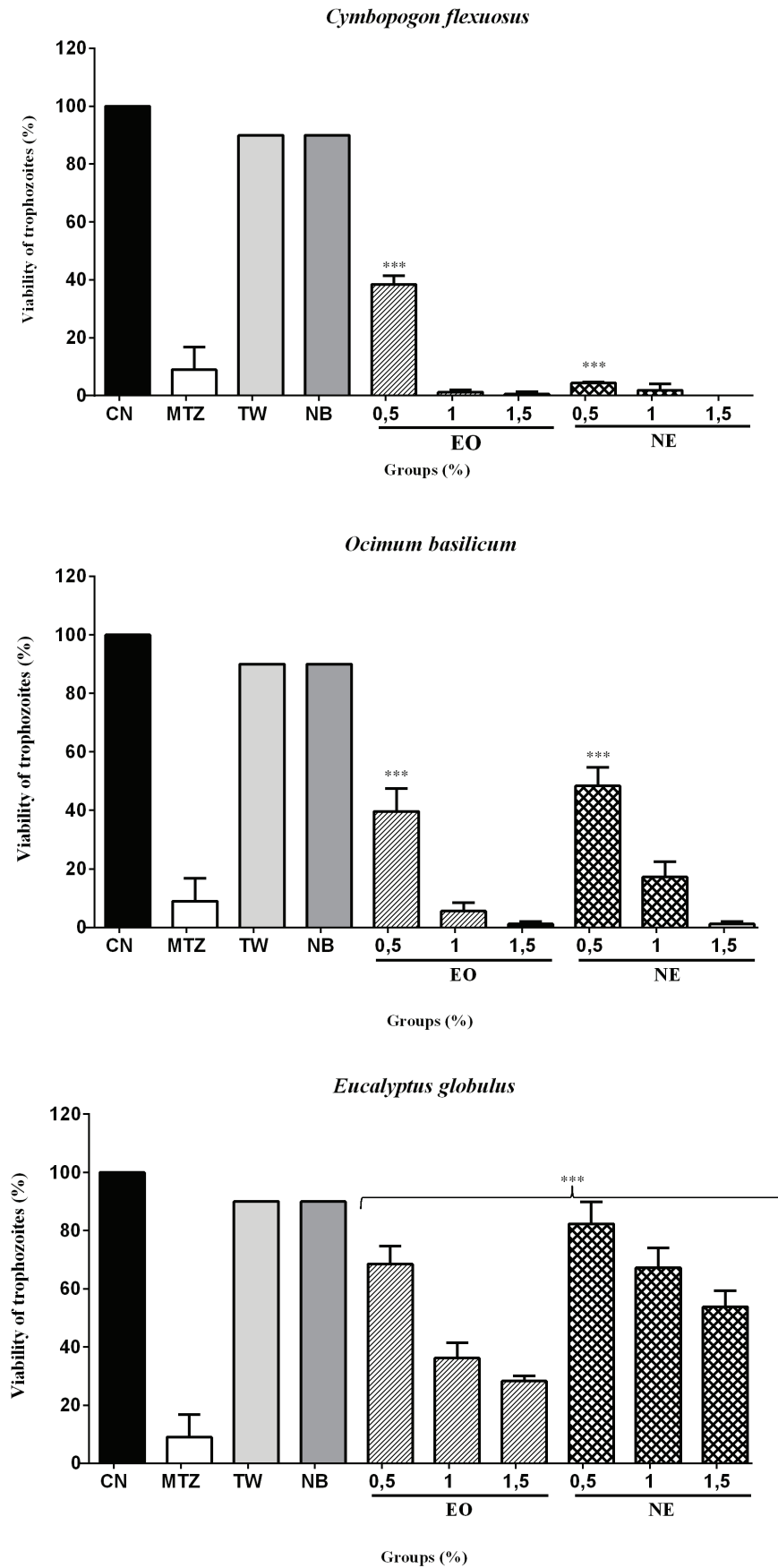
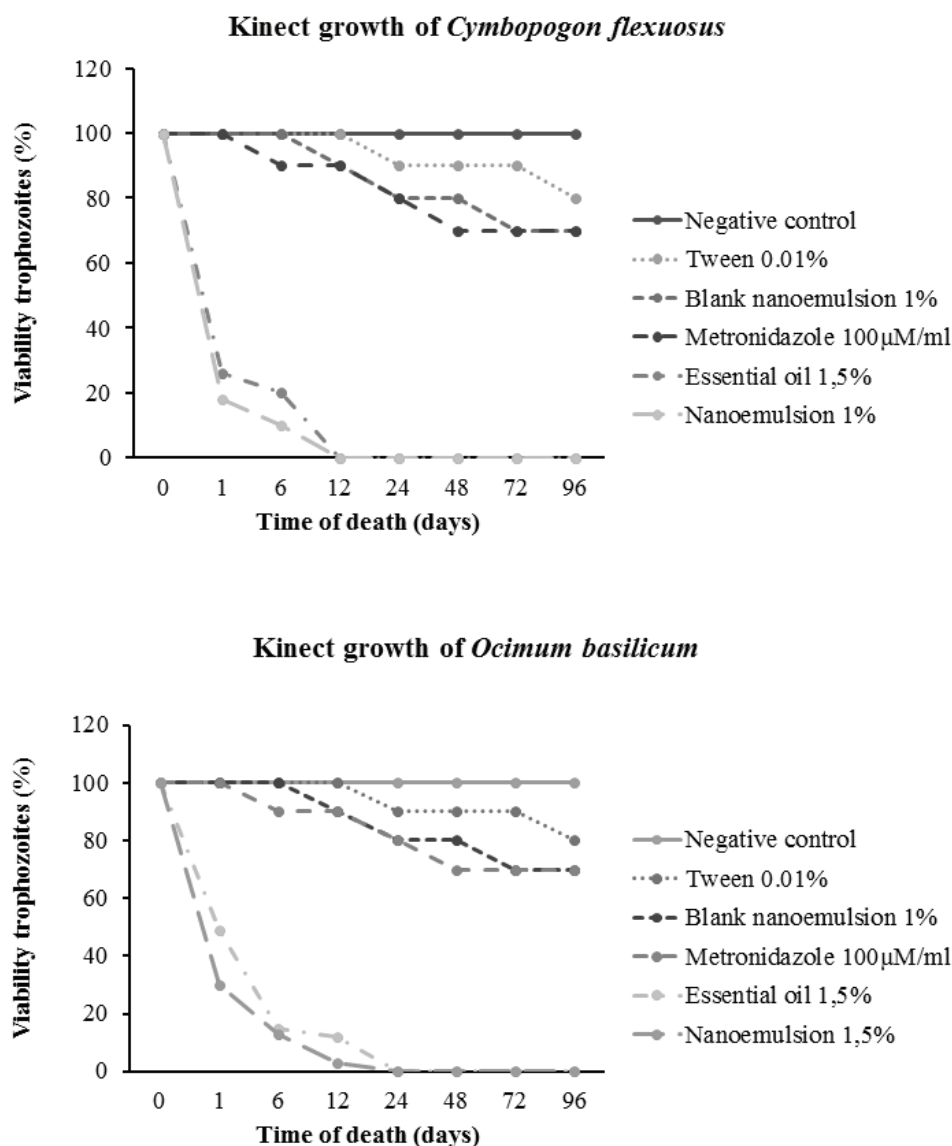
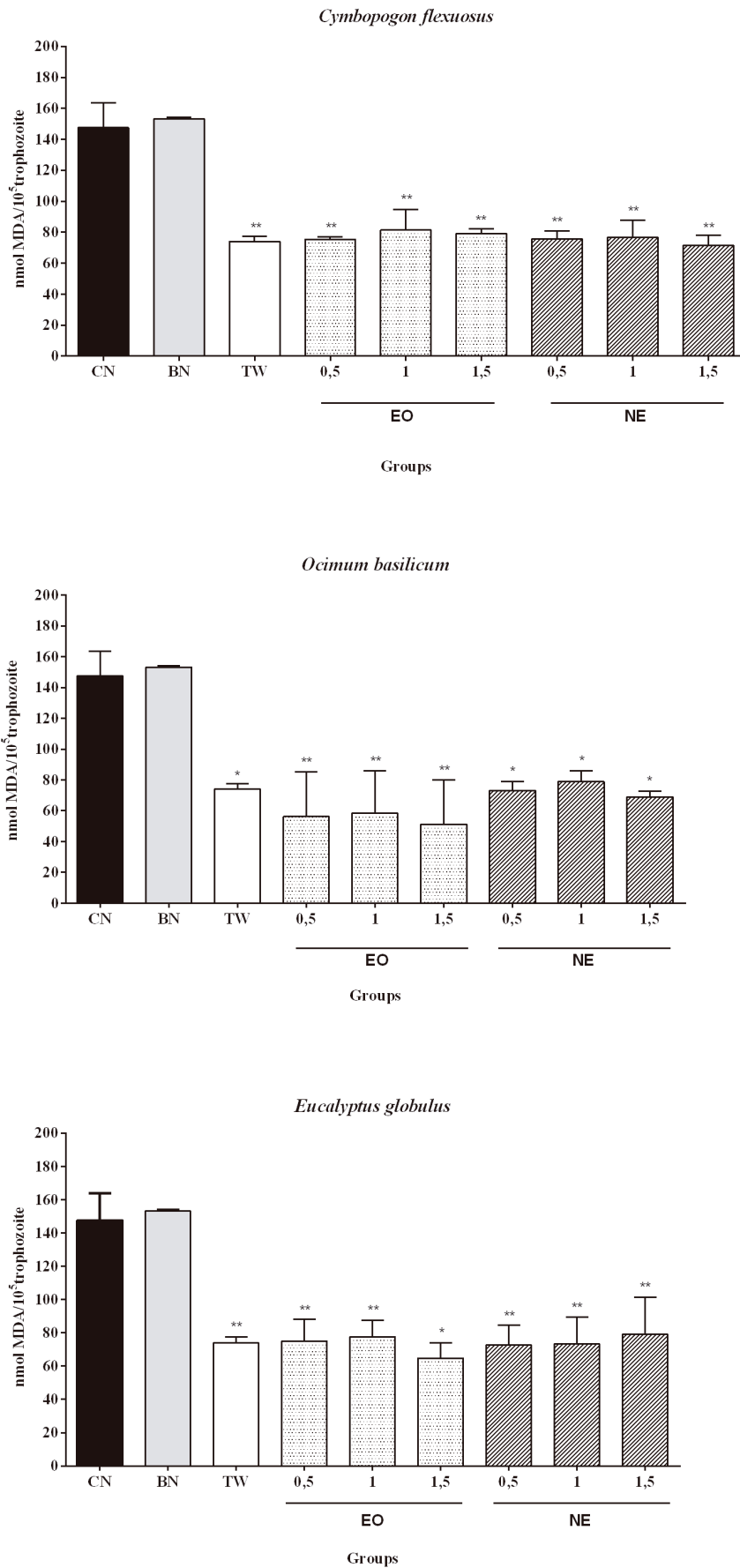


Figure 2 - Time of death of *Trichomonas vaginalis* isolate (MIC) after exposure to free oils (EO) and nanoemulsion (NE), respectively, of *Cymbopogon flexuosus* and *Ocimum basilicum* at 1h, 6h, 12h, 24h, 48, 72h and 96h.



The TBARS assay showed that trophozoites at negative control (NC) increased lipoperoxide levels. Nevertheless, when free EO or nanoemulsions containing these oils were added, as well as Tween 80, peroxidation activity was reduced, showing significant difference when compared to the NC, as shown in the graphic (Figure 3).

Figure 3 - TBARS assay on *Trichomonas vaginalis* trophozoites after treatment - free and nanostructured essential oils of *Cymbopogon flexuosus*, *Ocimum basilicum* and *Eucalyptus globulus*



DISCUSSION

Several reports over the years have shown *T. vaginalis* resistance when exposed to MTZ, even at low levels (DUNNE *et al.*, 2003; CUDMORE *et al.*, 2004; KIRKCALDY *et al.*, 2011). Studies which aimed to evaluate biological activity, like our research, emphasize the increase of the search for new metabolites with therapeutic value considering the large biodiversity within the Brazilian territory and the potential of natural products in the treatment of diseases (BASSO *et al.*, 2005). Our study found promising results using lemongrass and basil EO, in addition to the new data with nanoemulsion.

In this work, the compounds identified by gas chromatography for *C. flexuosus* corresponded to the literature. The lemongrass was composed mainly of an isomeric mixture of two compounds, alpha-citral (geranial) and beta-citral (neral) (KAKARLA; DEEPAK, 2009), and this is the first report of *anti-T. vaginalis* activity for *C. flexuosus*. In addition to a complex natural mixture, where the synergism between these chemical components could be linked to their promising pharmacological potential (BAKKALI *et al.*, 2008; GALINDO *et al.*, 2010). Thus, they act through different mechanisms of action in several targets within the cells, as in the cell proteins present in the cytoplasmatic membrane (BURT *et al.*, 2004).

Our study found higher trophozoites inactivation of *T. vaginalis* when treated with lemongrass in both presentations, as detected by Adukwu *et al.*, (2016) against four strains of *Acinetobacter baumannii*, including two multi-drug-resistant, which showed susceptibility when exposed to *C. flexuosus*, being an alternative for resistant infections. The higher bioactivity of nanostructured clove EOs when compared to free oil and amikacin, was also evidenced in the present study with lemongrass incorporated into nanostructures, presenting similar activity to free oil and MTZ, in lower concentration (ANWER *et al.*, 2004). This highlights the potential of natural products associated to nanoemulsion droplets, enhancing the biological properties of the components to the target.

Nanotechnology has been used as an alternative to improve the activity of EOs. In our study, this formulation probably contributed for a higher cellular absorption and bioefficacy, avoiding the decomposition of these oils due to environmental factors, thus maintaining stability and decreasing volatility, which can justify the higher activity presented by lower concentrations of lemongrass nanoemulsion when exposed to trophozoites (BILIA *et al.*, 2014). This nanoemulsion was able to reduce the MIC of lemongrass, when compared to free oil, as observed with a nanostructured drug in another study (BALDISSERA *et al.*, 2013; FUENTEFRIA *et al.*, 2014; BAJERSKI *et al.*, 2016). The protection offered by nanostructures to bioactive content enhance the delivery to target, resulting in more effectiveness than other conventional forms (LOVELYN *et al.*, 2011).

Studies have suggested that the use of drug system delivery in nano size may increase passive cellular absorption, probably through the association of porins and phenolic compounds. This interaction promotes cell breaking due to leakage of ions and others cell contents, increasing the

bioactivity and consequently the therapeutic efficacy of oils when nanostructured (DONSI *et al.*, 2012; SALVIA-TRUJILLO *et al.*, 2015), which justifies the better activity of lemongrass nanoemulsion observed in our study against *T. vaginalis*.

Nanostructured systems are prepared from different materials with dimension ranging from 0.1 to 500 nm. This size and biocompatibility could lead to better penetration into the membrane of the trophozoites and facilitate EO activity, which could explain the significant inhibitory activity observed in our tests. The surface charge of nanoemulsions droplets, also known as zeta potential, is the electrical characteristics of the delivery system drug (MCCLEMENTS *et al.*, 2011). Controlling the charge is a way to control the interactions with the infectious agent, since the microorganisms have the opposite charges to the nanoemulsions, and when in contact with the oil incorporated into these droplets, there will be electrostatic attraction (ZIANI *et al.*, 2011; CHANG *et al.*, 2015). The electrostatic attraction could be discarded in this study, because the nanoemulsion has negative charge and the trophozoites membrane is made up mainly by sialic acid, which contributes to the negative charge of the protozoan (SILVA-FILHO *et al.*, 1986).

The performance of basil against *T. vaginalis* is in accordance with the literature. The same growth inhibitory ability of the EO observed in our research was reported earlier. That study suggests that the antiprotozoal activity observed is probably related to the capacity of basil to inhibit the proteolytic activity of cysteine proteinase, an enzyme present in the life cycle of several protozoan, and the same may occur with *T. vaginalis* (ELDIN *et al.*, 2015). In our study, the growth inhibition was dose and time dependent and no statistically significant difference was detected when compared to MTZ.

However, there are no reported studies using nanoemulsions containing *O. basilicum* with anti-*Trichomonas* activity for comparison. This is the first time that the activity of this nanostructured oil was reported against this protozoan. This activity can also be justified by the characteristics of the EO, which has low density and hydrophobicity that facilitate the delivery of active compounds into specific target cells in parasites (MONZOTE *et al.*, 2012). The literature states that basil oil is composed of terpenes (linalool and geraniol) and phenylpropanoids (methyl chavicol, eugenol, methyl eugenol and methyl cinnamate). This varying concentration or presence of major compounds between the species can be justified by environmental factors and the geography of the place where the plant was obtained (ÖZCAN *et al.*, 2005; PANDEY *et al.*, 2014).

Anti-*trichomonas* activity revealed that no growth was observed after 24h of incubation of lemongrass and basil. The mechanism of action of EOs against microorganisms has not been understood yet. Studies have deduced that the bioactive compounds penetrate the phospholipid bilayer after attaching to the membrane, acting directly in the synthesis of the component cell promoting a structural destabilization and consequent death of the parasite (CHOUHAN *et al.* 2017). The same ability of these oils to inhibit the growth of trophozoites was also observed by Baccega *et al* (2020) against *T. gallinae*, confirming the antiprotozoa activity regarding this species.

The promising pharmacological potential of EOs against parasites could be linked to major compounds or to the synergism within this complex natural mixture, acting through different mechanisms of action in several targets within the cells, such as the proteins present in the cytoplasmic membrane (BURT *et al.*, 2004; BAKKALI *et al.*, 2008). The literature suggests that this relationship of bioactive compounds contributes to avoid the emergence of new resistant strains, due to the different receptors that the oil can affect during all stages of the lifecycle of a parasite (ANDRÉ *et al.*, 2018).

Basil and lemongrass oils were identified in this study as potent antioxidants with ability to improve lipid and antioxidant status. EOs can be considered good sources of natural compounds with significant antioxidant activity, which can also be attributed to their main constituents. The capacity of phenolic compounds of donating hydrogen or an electron and removing oxygen from free radicals is responsible for this biological property (SINGH; NISHI, 2015). The reduction of lipid peroxidation observed for the treatments with free and nanostructured oils is confirmed by earlier reports. It is well known that lemongrass is a source of bioactive compounds, flavonoids and vitamins which could inhibit the chain reactions of lipid oxidation, thus justifying the antioxidant activity obtained (IBRAHIM; FERIAL, 2013).

CONCLUSIONS

Lemongrass and basil are promising alternatives for the treatment of trichomoniasis. Furthermore, lemongrass showed that the incorporation of oils into nanoemulsions is an attractive option in the developing of new biologically active natural drugs, to promote therapeutic efficacy in the treatment of this sexually transmitted infection.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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