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Antimicrobial and anti-biofilm activities of Alpinia zerumbet (Pers.) B.L. Burtt & R.M. Sm. essential oil against Corynebacterium ulcerans

Pâmela Ruth Santos Viana[I], Denes Sousa Leite[II], Victor Elias Mouchrek Filho[III], Dionney Andrade de Sousa[IV], Ana Luíza de Mattos-Guaraldi[V], Márcia Barros Alves[VI], Wellyson da Cunha Araújo Firmo[VII], Priscila Soares Sabbadini[VIII]

ABSTRACT

Multiresistant strains of Corynebacterium ulcerans highlight the necessity of researches in new drugs and the pharmacological use of essential oils (EO) could be a therapeutic alternative. Thus, this study aimed to evaluate the antibacterial activity and the effect of subinhibitory concentration of AZEO on bacterial morphology and biofilm formation on polystyrene surface by C. ulcerans isolated in dogs. The AZEO was obtained by steam distillation from the leaves of the plant. The phosphomolybdenum and hemolysis inhibition tests were made to analyse, respectively, the antioxidant and toxicity activity. It was determined the minimum inhibitory and bactericide concentrations (MIC and MBC) of AZEO on C. ulcerans strains and from these results tests were made with subinhibitory concentrations of AZEO. The inactivation of phosphomolybdenum complex by AZEO was lower than 1% and the hemolytic capacity was low. The antimicrobial study results indicated that AZEO inhibited growth of both tested microbial strains. Bacterial filamentation was observed in AZEO presence, as well as bacterial autoaggregation. There was significant difference in biofilm inhibition. We suggest that A. zerumbet may represent an alternative therapy to control C. ulcerans-induced bacterial infections.
Keywords: Antioxidant; Filamentation; Autoaggregation

1 INTRODUCTION

The popular use of medicinal plants is fundamental to understand its therapeutic potential. Phytotherapics have its medicinal uses defended for centuries by positive results found in many diseases, though phytotherapics are few studied and have no its chemical components known. This fact awakens the interest of researches in studies that are related to plants pharmacology and phytochemical, improve the knowledges about natural medicine (FIRMO *et al.*, 2011).

[I] Universidade CEUMA, São Luís, Brasil - ruthvianna789@gmail.com
[II] Universidade CEUMA, São Luís, Brasil - denessousa27@gmail.com
[III] Universidade Federal do Maranhão, São Luís, Brasil - victor.mouchreck@ufma.br
[IV] Universidade Federal do Maranhão, São Luís, Brasil - dionney.andrade@ufma.br
[V] Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brasil - aguaraldi@gmail.com
[VI] Universidade CEUMA, São Luís, Brasil - marcia_barros16@hotmail.com
[VII] Universidade CEUMA, São Luís, Brasil - well_firmo@hotmail.com
[VIII] Universidade CEUMA, São Luís, Brasil - priscila.sabbadini@ceuma.br

The pharmacological studies aim to certify the collateral effects of the products obtained from plants, relating them to tested doses *in vitro* and *in vivo* assays, as well as verifying the efficacy and the toxicity of herbs of popular and commercial use (LAPA *et al.*, 1999). However, it is necessary the researches of biological activities of medicinal plants are increased in order to provide details that could serve as basis to obtain new scientific knowledges (SILVA; QUADROS; MARIA NETO, 2015).

Alpinia zerumbet (Pers.) B.L. Burtt & R.M. Sm., a plant transported from Asia to Brazil at the time of its discovery, belong to *Zingiberaceae* family and it is found in scientific literature as synonym of *Alpinia speciosa* K. Shum, *Costus zerumbet* Pers., *Languas speciosa* Small and *Zerumbet speciosum* J. C. Wendel (LORENGI; SOUZA, 2001). In southeast and northeast of Brazil, where it is commonly known as Colônia, the leaves of *A. zerumbet* are used in infusion as diuretic, antihypertensive and antiulcerogenic (MATOS, 1998). The antimicrobial activity proven to *A. zerumbet* essential oils (AZEO) varies according to its composition (WATTIEZ; STERNON, 1942).

In 1986, it was first described the *Corynebacterium* gender, a group of bacteria that presented similarity with gram-positive diphtheroid bacillus (LEHMANN; NEUMANN, 1986). The specie that stand out in this gender is *Corynebacterium diphtheriae,* etiologic agent of diphtheria (BURKOVSKI, 2014). However, the reports of respiratory diphtheria-like illness caused by toxigenic *Corynebacterium ulcerans,* mainly in Europe, that exceed the cases caused by *C. diphtheriae,* are increasing (HACKER *et al.*, 2016; OTHIENO *et al.*, 2019).

The history of human's infections caused by C. *ulcerans* is related to raw milk consumption or the contact with domestic animals (HOGG *et al.*, 2009). This pathogen is a commensal of domestic animals and wild animals, which could serve as reservoir to zoonotic transmission of the bacteria (DIAS *et al.*, 2011; CONTZEN *et al.*, 2011; SIMPSON-LOUREDO *et al.*, 2014). It had already been described by ribotyping that the same strain of this specie was presented in both a diphtheria patient and her dog (LARTIGUE *et al.*, 2005).

Mattos-Guaraldi *et al*. (2008) described the first *C. ulcerans* strain producing a diphtheria-like toxin isolated from an elderly woman with a fatal pulmonary infection and a history of leg skin ulcers in the Rio de Janeiro, Brazil. In addition to diphtheria toxin, phospholipase D is considered a virulence factor of *C. ulcerans*. *C. ulcerans* showed affinity to fibrinogen, fibronectin and type I collagen (SIMPSON-LOUREDO *et al*., 2019) and moderate susceptibility to some antibiotics (MATTOS-GUARALDI *et al*., 2008; SIMPSON-LOUREDO *et al*., 2014).

Little information is available concerning the effects of plants on properties of *Corynebacterium* spp. Hence, the present study aimed to evaluate the antioxidant, antibacterial and antibiofilm activities of *A. zerumbet* on *C. ulcerans* isolated from dogs.

2 MATERIALS AND METHODS

2.1 Plant material and hydrodistillation procedure

The leaves of *A. zerumbet* were collected in the morning in São Luís, Brazil (2°33'01.7"S 44°13'32.9"W) in April 2017. The plant material was submitted to drying in ambient temperature and the essential oil (EO) was hydrodistillated using a Clevenger-type apparatus (ADAMS, 2007). Sterile dimethyl sulfoxide [DMSO; 1% in phosphate buffered saline (PBS)] was used as an oil solubilizer. Yield of the essential oil (%) was calculated on the basis of the moisture-free material.

2.2 Phosphomolybdenum complex assay

Spectrophotometric evaluation of antioxidant activity through the formation of a phosphomolybdenum complex was carried out according to Emerenciano *et al*. (2013). Sample solutions (0,4mL) were combined in an tube with 4mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of aqueous solutions of each was measured at 680nm against a blank. Ascorbic acid and AZEO were tested at 200µg/mL concentration. The antioxidant activity was expressed

as the absorbance of the AZEO in relation to ascorbic acid, considering the value of its absorbance was 100% antioxidant action. This assay was performed in triplicate and repeated at least twice.

2.3. Hemolytic activity

The test was made with red blood cells obtained from a human voluntary and healthy (with no recent history of antibiotic therapy or with anti-inflammatory drugs and/or infectious diseases or inflammatory three weeks before the collect of the sample). The study was reviewed and approved by the Human Research Ethics Committee of the Universidade CEUMA, Brazil (CEP-UNICEUMA) under protocol number 1.732.522 and was performed in accordance with the Declaration of Helsinki 1975, as revised in 2008.

Hemolytic assays were performed according to the method previously described by Yang, Sun and Fang (2005) and Oliveira *et al*. (2011), modified. Briefly, 0,5mL aliquots of human erythrocytes 1% in saline solution (0.9%) were added to 0.5mL of EO in concentrations ranging from 1000 to 50µg/mL. After preparation, the solutions were incubated for 60 minutes at 37 ºC and centrifuged at 3000rpm for 5 minutes. From the supernatant fluid, 100µL were transferred to a flat bottom microtiter plate and absorbance was measured using a spectrophotometer (550nm). To eliminate the EO inference in absorbance, control solutions (white) were prepared without addition of the red blood cells solution. Total and no hemolysis were achieved with Triton X-100 (1mg/mL) and saline plus red blood cell suspensions, respectively. The level of percentage hemolysis was calculated according to the following formula:

$$\% \text{ Hemolisys} = [(At - An) \times 100]/(Ac - An)$$

Here: At is the absorbance of test sample.

An is absorbance of the negative control (saline).

Ac is the absorbance of the positive control (Triton X-100).

2.4 Antibacterial activity

2.4.1 Origin of bacterial strains and culture conditions

The microorganisms (Table 1) were kindly give by Laboratory of Diphtheria and Corynebacteria of Clinical Relevance/Universidade do Estado do Rio de Janeiro, Brazil, and are stocked in GC-glicerol 20% in Laboratory of Bacterial Respiratory and Systemic Diseases/Universidade CEUMA, Brazil.

To assays, the microorganisms were cultivated for 24-48hours/37°C in Trypticase Soy Broth (TSB, Difco), Trypticase Soy Agar (TSA, Difco) and/or sheep blood agar and tellurite chocolate agar medium (SABBADINI *et al*., 2010).

Table 1 – Origin of partially studied *Corynebacterium ulcerans* strains isolated from dogs in Pernambuco, Brazil

Strains	Origin	Isolation site	tox gene*
2649	Asymptomatic dog	Nasopharynx	-
2652	Asymptomatic dog	Nasopharynx	-

*, diphtheria toxin; -, negative

2.4.2 Minimal Inhibitory and Bactericidal Concentrations

The antimicrobial activity of AZEO was determined by the microdilution. Briefly, each strain was grown on TSA plates at 37°C for 48h, suspended in saline solution (~1.5 × 10^8 CFU/mL) and diluted in TSB culture medium (ratio 1:10). For the determination of minimum inhibitory concentrations (MICs), aliquots of bacterial suspension were incubated in TSB containing EO at different concentrations (60–2.000µg/mL). Samples were then incubated for 48h at 37°C. After the incubation period, 30µL of resazurin (0.03%) working solution was added to each well. The microtiter plates were then incubated again for 30 minutes at 37°C. The results were reported visually. Color changes from blue to pink indicated reduction of resazurin which meant bacterial growth. The MIC value was determined as the lowest concentration of the test solution which prevented full color changes of resazurin.

Serial dilutions of penicillin (0.015–256µg/mL) were used as positive controls. The effect of the addition of DMSO 1% as an oil solubilizer on MIC was investigated and it was found that this concentration did not interfere with bacterial growth.

For determining the minimum bactericidal concentrations (MBCs), just after the MIC experiments, the cultures were seeded on TSA medium and incubated for 48h at 37°C. The MBC corresponded to the lowest concentration of the compound to which no viable bacteria was observed (SANTURIO *et al.*, 2007). Experiments were performed twice in triplicate.

2.4.3 Bacterial morphology

The experimental model of bacterial morphology elaborated based on a system used by Gomes *et al.* (2013) with modifications. Microorganisms [Optical Density (OD) 0.4; 570nm] were grown in TSB medium with or without subinhibitory concentrations (subMIC) of the AZEO (MIC/2 and MIC/4). After 48 hours of incubation at 37ºC, all strains were observed after Gram staining by light microscopy.

2.4.4 Anti-biofilm activity

Biofilm formation was quantified according to the method previously described by Stepanovic *et al.* (2004). Briefly, aliquots of bacterial suspensions in TSB with OD_{570} 0.2 were applied per well into 96 well cell culture plate with or without subMIC of the AZEO (MIC/2 and MIC/4). DMSO 1%-treated bacteria and TSB without bacteria were used as positive and negative controls, respectively. After 48 hours at 37ºC, the content of each well was aspirated and washed twice with PBS (pH 7.2, 0.01M). The remaining attached bacterial cells were fixed with 99% methanol and were stained with 2% crystal violet. The bound dye was then solubilized with 160µL 33% glacial acetic acid and absorbance was taken at 570 nm using an enzyme immunosorbent assay reader (THERMOLAB). Mean absorbance values of each sample were calculated and compared with the mean values of controls. Experiments were performed twice in triplicate.

2.5 Statistical Analyses

GraphPad Prism 6 program was used for data analyses. The statistical comparison between groups were analyzed using Turkey test. A $p<0.05$ was considered significant.

3 RESULTS

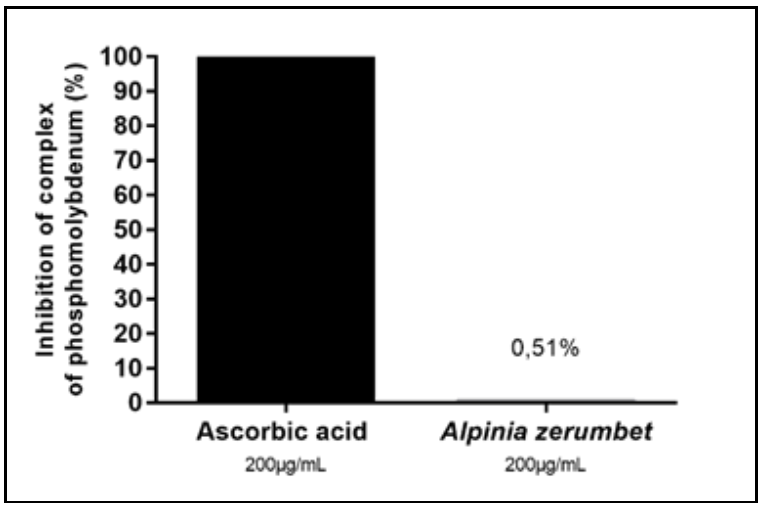
3.1 *A. zerumbet* essential oil yield

The essential oil of *A. zerumbet* showed yield of 0.31%.

3.2 Evaluation of the antioxidant capacity

A low antioxidant activity of 0.51% was obtained for the AZEO as compared to ascorbic acid positive control after the phosphomolybdenum assay (Figure 1).

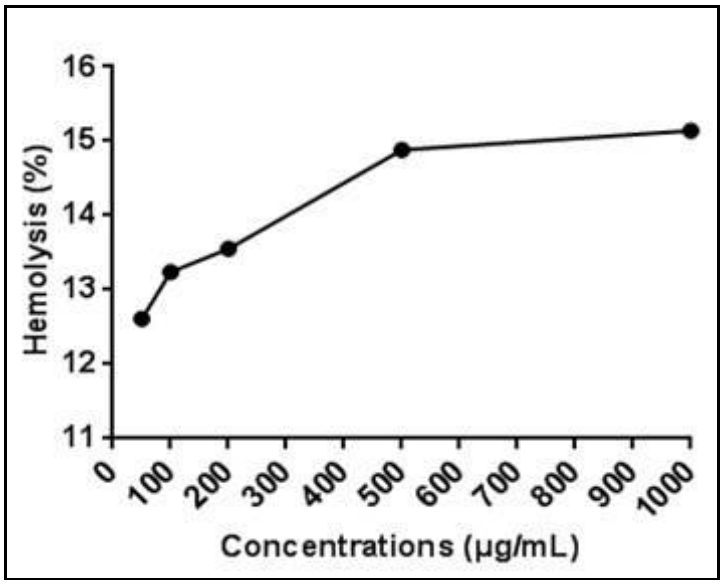
Figure 1 - Antioxidant activity of the essential oil of *Alpinia zerumbet* by the method of the phosphomolybdenum complex



3.3 Hemolytic activity

The hemolytic activity of the AZEO was investigated *in vitro* in human erythrocytes. The hemolytic percentage 12.61%, 13.24%, 13.55%, 14.88% and 15.14% were obtained for a dose of 50µg/mL, 100µg/mL, 200µg/mL, 500µg/mL and 1000µg/mL, respectively (Figure 2).

Figure 2 - Effect of essential oil of *Alpinia zerumbet* on hemolytic activity



3.4. *A. zerumbet* inhibits the growth of *C. ulcerans*

The antibacterial activity of AZEO was highest against *C. ulcerans* strain 2652 (MIC 250µg/mL) (Table 2). The MBC could not be determined with the concentrations tested.

Table 2 - Minimum inhibitory and bactericidal concentrations of *Alpinia zerumbet* essential oil on *Corynebacterium ulcerans*

Strains	MIC1		MBC2
	AZEO3	Penicillin 0.03	AZEO
2649	500	1	ND4
2652	250	1	ND

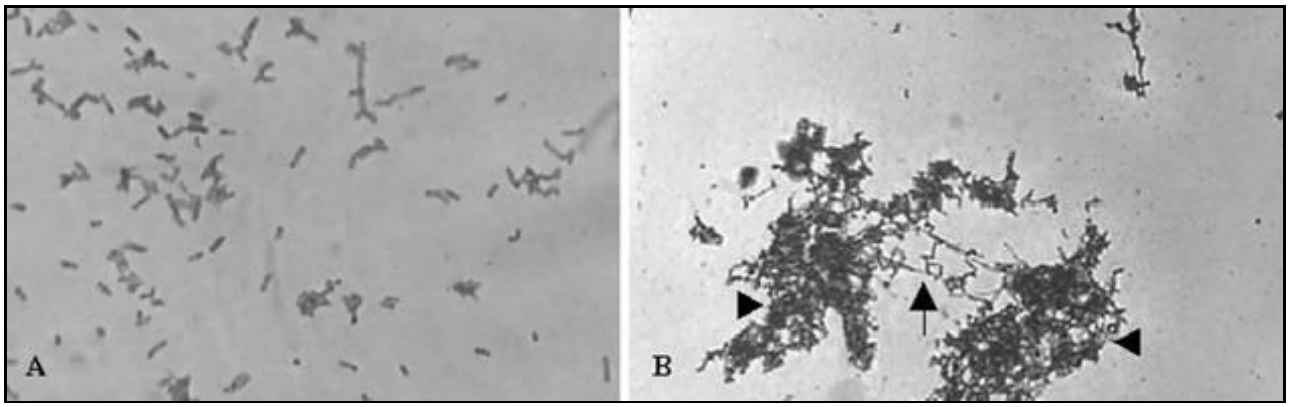
[1]MIC, Minimum Inhibitory Concentration is expressed in µg/mL. [2]MBC, Minimum Bactericidal Concentration. [3]AZEO, *Alpinia zerumbet* essential oil. [4]ND, Not determined with the concentrations evaluated. Penicillin was positive control.

3.5 *A. zerumbet* subMIC effect on bacterial morphology

C. ulcerans strains 2649 and 2652 showed an increase in bacterial autoaggregation after growth in the presence of subinhibitory concentrations (MIC/2 and MIC/4) of AZEO, however *C. ulcerans* strain 2652 showed a greater increase in aggregation (Figure 3).

Morphological changes suggesting bacterial filamentation were also observed in *C. ulcerans* strain 2652 (Figure 3). Microorganisms cultured in the presence of 1% DMSO did not present morphological alterations or increase in the bacterial grouping.

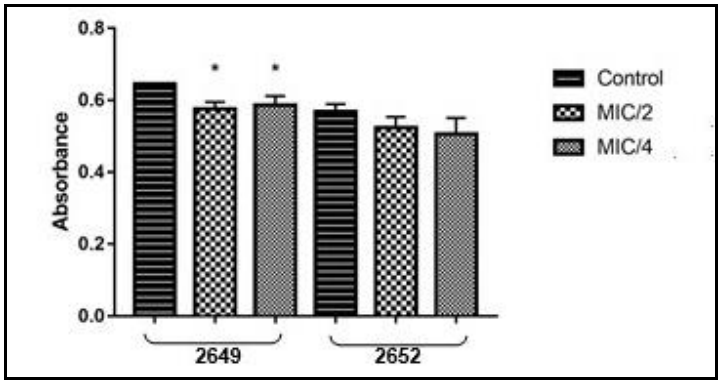
Figure 3 – Photomicrographs of Gram-stained cells of C. ulcerans strain 2652 grown in the absence (control) (A) or in the presence of MIC/4 of Alpinia zerumbet essential oil. Arrow and arrow heads indicate bacterial filamentation and grouping, respectively. Magnification x1000



3.6 *A. zerumbet* subMIC decreases biofilm formation

We attempted to analyze the effects of subMIC of AZEO (MIC/2 and MIC/4) on the ability of *C. ulcerans* strains 2649 and 2652 to form biofilm. As depicted on Figure 4, AZEO decreased biofilm formation by these bacteria at both tested concentrations. However, the correlation test showed significant difference ($p<0.05$) only for the *C. ulcerans* 2649 strain. It was observed that 1% DMSO did not influence in the biofilm formation in analyzed strains.

Figure 4 – Effect of Alpinia zerumbet essential oil (MIC/2 and MIC/4) on biofilm formation in Corynebacterium ulcerans strains. *p<0.05, compared with DMSO 1%-treated bacteria (Turkey test). Experiments were performed twice in triplicate. Each bar represents mean ± SD. The absorbance values were obtained in nm.



4 DISCUSSION

It is necessary to understand the plant potential and their chemical components as agents to be used in the prevention and/or treatment of diseases, especially infectious. Thus, the development of studies with vegetal species is fundamental, particularly in Brazil, where the biodiversity is significant (RAMESHA *et al.*, 2011).

Oil used in this study showed low yield (0.31%). According to Santos *et al* (2012), the lowest values of AZEO were observed in the morning, followed by an increase in the afternoon, reaching the maximum concentration at 14:33 h (0.48%). Essential oils generally reach their highest contents in the morning (MARCHESE; FIGUEIRA, 2005), however, with the advancement of the researches, different responses have been verified, depending on each species. Moreover, distillation time has been shown to have an influence on essential oil yield and composition of crops such as peppermint, lemongrass, palmarosa (CANNON *et al.*, 2013) and lavender (ZHELJAZKOV *et al.*, 2013).

Several studies have been presented about the antioxidant capacity of Brazilian plants (DE MORAIS *et al.*, 2006; FIRMO *et al.*, 2011; VIANA *et al.*, 2017). Despite the low antioxidant activity found in AZEO in this study, evaluated by phosphomolybdenum complex methodology, Tu and Tawata (2015) mentioned a strong AZEO antioxidant potential on 2.2-diphenil-1-picrilhydrazyl free radicals. Methanolic extracts of *A. zerumbet* presented antioxidant activity equivalent to ascorbic acid (WONG; LIM; OMAR, 2009). Also, in Wong, Lim and Omar (2009) study, polyphenols were found in methanolic extracts of *A. zerumbet*. The polyphenols are antioxidants and act as donor of hydrogen and reducer of reactive oxygen species (BALASUNDRAM; SUNDRAM; SAMMAN, 2006).

According to Sucupira *et al.* (2012), due to the diversity of methods with varied fundamentals and interferences, the comparison of the results of antioxidant activity becomes difficult. Moreover, the environmental conditions (light, humidity, temperature and predators) may represent an important differential factor in the production of secondary metabolites in the plant, which could directly reflect on the

antioxidant capacity (GOBBO-NETO; LOPES, 2007). Kuraya *et al*. (2017) revealed that the antioxidant activity and yield of AZEO differed significantly between individual plants and collecting seasons.

The emergence of antimicrobial resistant microorganisms has increased the search for new therapeutic agents, encouraging the researches with medicinal plants, since many of them may have antimicrobial properties. However, it is fundamental medicinal plants and its extracts, or its essential oil be considered safe, without sensitizing manifestations that could endanger the user health. Lysis of erythrocytes cells is easily obtained by measuring the release of hemoglobin, constituting a good tool for toxicity studies. Determination of hemolysis activity is quick, reproducible, and inexpensive (OLIVEIRA *et al*., 2011; MAPFUNDE; SITHOLE; MUKANGANYAMA, 2016).

Here, the hemolytic effect of AZEO is seen to increase with the incresing concentrations of the oil. Thus the AZEO have shown dose dependent hemolytic activity. Ralph *et al*. (2009) through testing for hemolytic activity rated the degree of in vitro toxicity according to the observed mortality rate: 0 to 9% = non-toxic, 10 to 49% = slightly toxic, 50 to 89 % = toxic; 90 to 100 % = highly toxic. In this study, AZEO was reported to possess weakly hemolytic activity towards human erythrocytes.

The hemolytic activity of plants is related to their phytochemical composition (ZOHR; FAWZIA, 2014). Some studies have demonstrated the presence of saponin in the AZEO (COSTA *et al*., 2015). Saponins induce hemolysis through interaction with erythrocyte cell membrane components, causing membrane deformation and consequent disruption (ARABSKI *et al*., 2012; CARVALHO; OLIVEIRA, 2012).

In an in vitro model using human leukocytes, the AZEO did not induce genotoxicity at concentrations among 50-300µg/mL (CAVALCANTI *et al*., 2012). Acetone and methanolic extracts tested in carcinogenic cell lines did not presented no significant toxicity (CÔRREA; COSTA, 2008). The AZEO (400mg/kg) did not show a mutagenic profile in peripheral blood cells and bone marrow in mice (CAVALCANTI *et al*., 2012).

The EO obtained from *A. zerumbet* rhizome showed antibacterial activity on several Gram positive and negative species as: *Streptococcus mutans*, *Staphylococcus aureus*, *Micrococcus luteus*, *Salmonella tiphy* and *Pseudomonas aeruginosa*

(INDRAYAN; TYAGI; AGRAWAL, 2010). The chloroform extracts of *A. galanga* was identified with the MIC and MBC values against *S. aureus* at 128 and 256µg/mL, respectively (VORAVUTHIKUNCHAI; PHONGPAICHIT; SUBHADHIRASAKUL, 2005).

We have also demonstrated that AZEO was active against both strains of *C. ulcerans*. MBC could not be defined with the concentrations evaluated. However, it was required a lower AZEO concentration to inhibit *C. ulcerans* strain 2652 (MIC value of 250µg/mL) in comparison with strain 2649 (MIC value of 500µg/mL). The MIC value of penicillin (1µg/mL) was much lower in comparison to MICs of AZEO for the test organisms. From the study it was clearly observed that AZEO have anti bacterial effect but is not potent like antibiotic penicillin.

Various in vitro experiments have established the fact that a combination of plants and antibiotics possess a synergistic effect, which results in a significant decrease in levels of MIC for the antibiotics. The combination may be helpful in the preclusion of the emergence of resistant bacteria and reducing the drug toxicity (CRISTO *et al*., 2016; RAFIQ *et al*., 2017).

Studies using low concentrations of antimicrobial compounds have shown that, besides these concentrations did not kill the bacteria, they can modify the physical or chemical structure of their cell wall, which may impact, for example, on motility, adhesion and toxin production (SUBRT; MESAK; DAVIES, 2011; GOMES *et al*, 2013).

Here, the growth of *C. ulcerans* strain 2652 in the presence of subMIC of AZEO induced morphological alterations which suggest filamentation. Sutton *et al*. (2011) reported that subMIC of penicillin generated during treatment of *C. diphtheriae* infections may result in difficulties in diagnosis and/or treatment, as antimicrobial-mediated bacterial filamentation might resemble fungal hyphae, especially in body fluids where the antibiotics may reach sublethal concentrations. This misinterpretation during clinical diagnostic procedures may lead to inappropriate treatment.

We observed an increased in bacterial aggregation, especially for *C. ulcerans* strain 2652, after growth with AZEO subMIC. Phytochemical screening showed the presence of tannin in *A. zerumbet* (AL-ENAZI, 2018). Tannins have great ability to interact with

proteins and other macromolecules, which give them the capacity to interconnect different structures (CARVALHO; OLIVEIRA, 2012). Moreover, Gomes *et al*. (2013) reported that subinhibitory concentrations of antimicrobial agents enhanced cell-surface hydrophobicity of *C. diphtheriae.*

AZEO may present terpenes in its constitution (OLIVEIRA *et al*., 2017). According Dalleau *et al*. (2008), terpenes are substances that penetrate in lipid layers and that can alter cellular permeability and membrane fluidity, suggesting that these alterations cause superficial and morphological changes, reducing the cellular adherence and impacting in biofilm formation. In the present study, it was proved that AZEO subMIC inhibited the biofilm production on polystyrene surface for both *C. ulcerans* strains, although a greater bacterial aggregation was observed. Subinhibitory concentrations of crude hydroalcoholic extracts of *Pisidium guajava*, *Tithonia diversifolia* e *Anacardium occidentale* were also capable to significantly inhibit the biofilm production on polystyrene by *C. ulcerans* (FIRMO *et al*., 2018). *C. diphtheriae* strains showed an increase in biofilm production on polystyrene in the presence of subinhibitory concentrations of erythromycin (GOMES *et al*., 2013).

5 CONCLUSIONS

A. zerumbet essential oil demonstrated antibacterial properties as the capacity of promoting morphological alterations and reducing the biofilm production. Moreover, AZEO showed low toxicity proving to be safe for human use. The results strengthen the hypothesis of its potential to pharmacological use, since the plant has now been used for several diseases, as well as demonstrate the possibility of its use in the therapy of infections caused by *C. ulcerans.* In this regard active ingredient present in essential oil extracted from the leaves of *A. zerumbet* needs to be separated and purified for further study.

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