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USE OF COMPOUNDS FROM NEOTROPICAL PLANTS TO CONTROL DISEASE-CAUSING BACTERIA, VIRUSES AND INSECTS

WELLINGTON DE SOUZA MOURA

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Tese de doutorado apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia - Rede BIONORTE, na Universidade Federal do Tocantins, como requisito parcial para a obtenção do Título de Doutor em Biodiversidade e Biotecnologia.

Orientador(a): Prof. Dr. Raimundo Wagner de Souza Aguiar

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Moura, Wellington de Souza. **Uso de compostos de plantas neotropicais para controlar bactérias, vírus e insetos causadores de doenças.** 2021. 124f. (Doutorado em Biodiversidade e Biotecnologia). Universidade Federal do Tocantins, Gurupi, 2021.

RESUMO

Com o aumento de mortes causadas por microrganismos, seja pela resistência aos antibióticos, pelo surgimento de novas doenças e ainda pela alta transmissão por insetos vetores de doenças, surge a necessidade do desenvolvimento de tratamentos alternativos. Nesse sentido o uso de plantas oriundas de regiões neotropicais vem surgindo como forma promissora de bioprospecção de novas moléculas, em razão da alta biodiversidade dessa região. Plantas como *Siparuna guianensis* e *Chiococca alba* vem sendo estudadas por apresentarem em sua composição química compostos com ação contra esses alvos. O óleo essencial de *S. guianensis* possui em sua composição as moléculas de Germacrene B e D as quais apresentaram excelentes resultados nos testes de docking molecular realizados e no controle de bactérias patogênicas (Por exemplo, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* e *Streptococcus pyogenes*). As análises *in silico* indicaram alta afinidade entre alguns componentes principais do óleo essencial (por exemplo, Germacrene B) e sítios ativos de DNA e RNA polimerases bacterianas, o que indica possíveis prejuízos nos processos de replicação de células bacterianas patogênicas. Ao mesmo tempo, devido às suas características de alta volatilidade no controle de insetos vetores como o *Aedes aegypti* e *Culex quinquefasciatus*, o uso de técnicas de encapsulamento se faz necessário para aumentar seu potencial larvicida. Houve liberação contínua de óleo essencial durante o período de contato com as larvas, e a partícula OS1:3, na concentração de 1,667 mg/cm^3, ao longo de 16 dias apresentou mortalidade de 50%, aumentando assim a capacidade larvicida em relação ao óleo essencial in natura, em frente às larvas de 3º ínstar de *A. aegypti* e *C. quinquefasciatus*. Os bioensaios com as micropartículas ainda se mostraram seguros ao organismo não alvo zebrafish. Outro potencial alvo de plantas neotropicais se dá no controle do vírus Sars-CoV-2. O extrato metanólico de *C. alba* se mostrou promissor em testes de laboratório, apresentando alto índice de seletividade para controle de infecção de células Vero E6 contra Sars-CoV-2. Os estudos *in silico* demonstraram que a naringina e a vitexina se ligam aos receptores ACE2 x Spike com melhores valores de afinidade energética. Ensaios clínicos com humanos mostraram que o sachê e o chá preparado das raízes de *C. alba,* ao final do tratamento, apresentaram número significativo de pacientes com teste RT-PCR negativo e ainda a redução dos sintomas clínicos. Esses produtos não apresentaram reações adversas aos humanos, com resultados promissores contra Sars-Cov-2. Portanto podemos dizer que os resultados encontrados são promissores na bioprospecção de novas moléculas, demonstrando assim a importância do estudo de novas aplicações para as plantas oriundas da região neotropical.

Palavras-chave: Plantas neotropicais; micropartículas; Medicamentos fitoterápicos.

Moura, Wellington de Souza. **Use of compounds from neotropical plants to control disease-causing bacteria, viruses and insects**. 2021. 124f. (Doutorado em Biodiversidade e Biotecnologia). Universidade Federal do Tocantins, Gurupi, 2021.

ABSTRACT

With the increase in deaths caused by microorganisms, whether due to antibiotic resistance, the emergence of new diseases, and the high transmission by insect vectors of diseases, there is a need to develop alternative treatments. In this sense, the use of plants from neotropical regions has emerged as a promising form of bioprospecting for new molecules due to the high biodiversity of this region. Plants such as *Siparuna guianensis* and *Chiococca alba* have been studied for their chemical composition compounds that act against these targets. The essential oil of *S. guianensis* has the molecules Germacrene B and D in its composition, which showed excellent results in the molecular docking tests performed and in the control of pathogenic bacteria (i.e., *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes*). *In sílico* analysis indicated high affinity between some main components of the essential oil (e.g., Germacrene B) and active sites of bacterial DNA and RNA polymerases, which indicates possible damage to the replication processes of pathogenic bacterial cells. At the same time, with high volatility in the control of vector insects such as *Aedes aegypti* and *Culex quinquefasciatus*, the use of encapsulation techniques is necessary to increase their larvicidal potential. There was a continuous release of essential oil during the period of contact with the larvae, and particle OS1:3, at a concentration of 1.667 mg/cm³, presented mortality of 50% over 16 days, thus increasing the larvicidal capacity concerning the essential oil *in nature*, in front of the 3rd instar larvae of *A. aegypti* and *C. quinquefasciatus*. Bioassays with microparticles still proved safe for nontarget zebrafish. Another potential target of neotropical plants is the control of the Sars-CoV-2 virus. The methanol extract of *C. alba* showed promise in laboratory tests, showing a high selectivity index for controlling infection of Vero E6 cells against Sars-CoV-2. *In silico* studies have shown that naringin and vitexin bind to ACE2 x Spike receptors with better energy affinity values. Clinical trials with humans showed that the sachet and tea prepared from the roots of *C. alba*, at the end of the treatment, showed a significant number of patients with negative RT-PCR tests and a reduction in clinical symptoms. These products had no adverse reactions to humans, with promising results against Sars-Cov-2. Therefore, we can say that the results found are promising in the bioprospecting of new molecules, thus demonstrating the importance of studying new applications for plants from the neotropical region.

Keywords: Neotropical plants; microparticles; Herbal Medicines.

SUMMARY

1 INTRODUCTION

The increase in diseases caused by pathogenic microorganisms has become a major problem due to the number of deaths caused worldwide, which is due to the resistance of some of these microorganisms to drugs on the market (Raghubanshi et al., 2021), due to the emergence of new pathogenic microorganisms to humans (Loannidis et al., 2021) and by the spread of microorganisms by vector insects (Aliaga-Samanez et al., 2021). These situations are worrying the world population and encouraging the study of new alternatives for the control of bacteria, viruses, and disease-causing insects.

We can mention pathogenic bacteria that are resistant to antibiotics such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes*, which are agents of numerous diseases in humans and other warm-blooded animals (Rahman et al., 2018; Oliveira et al., 2019). We also have the Sars-CoV-2 virus, called the new coronavirus, causing COVID-19 (Yang et al., 2020; Riva et al., 2020) which has caused high mortality and morbidity and has spread rapidly, creating a huge impact on health systems around the world. Additionally, diseases caused by viruses such as dengue, yellow fever, chikungunya fever, and Zika are transmitted by the insect vector *Aedes aegypti* Linnaeus, 1762 (Diptera: Culicidae) (Lima-camara, 2016) and filariasis caused by the insect vector *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae) (Aguiar et al., 2015), therefore, plant compounds are emerging as an alternative for various treatments.

They have been used for many years in the most diverse applications, among which we can highlight their pharmacological use for medicinal purposes, which has increased the demand for biologically active and effective substances, especially in the control of microorganisms. What contributes to the increase in research is the fact that they are natural and biodegradable, generally have low toxicity in mammals, and because they act on several target molecules at the same time, thus becoming key substances in the search for new medicines, as they have several advantages, especially when compared to synthetic drugs (Negri et al., 2012).

The neotropical region is an area with great biodiversity; however, neotropical plants are still poorly studied, and are still unknown or poorly understood, mainly regarding their biotechnological applications (Turchetto-Zolet et al., 2013; Tinoco et al., 2015). Current bioprospecting procedures allow efficient discovery of new substances and from these to develop new bioproducts, thus adding value to biodiversity (Astolfi Filho et al., 2014). The Neotropics occur from Central Mexico to southern Brazil, including Central America, the Caribbean islands, and almost all of South America (Conservancy, 2005; Antonelli & Sanmartin, 2011), and stands out for having the largest number of plants and animals in the world (Tundisi & Tundisi, 2008).

Among the plants from the neotropical region studied, we can mention *Siparuna guianensis* Aublet (Siparunaceae), a plant popularly known as "negramina", belonging to the Siparunaceae family with several reports in the literature about its medicinal use, being used as a topical bath for symptoms of sinusitis, fever, rheumatism, migraine, flu, body aches and malina (Valentini et al., 2010). Another plant that has been widely studied is the species *Chiococca alba* (L.) Hitchc. (Rubiaceae) known by folk medicine in the central and southern parts of Brazil, Peru, French Guiana, Central America, and Florida (USA), popularly known as “caninana” in Brazil (Gazga, 2004).

In this sense, the present work sought to find alternatives with the use of compounds from these neotropical plants, identifying active molecules, the mechanism of action of these compounds in receptors, the elaboration of active particles, and the development of products to control bacteria, viruses, and insects causing diseases.

2 OBJECTIVES

This work aims to study of the use compounds from neotropical plants to control disease-causing bacteria, viruses and insects.

2.1 Specific objectives

- Use of *S. guianensis* essential oil to control pathogenic bacteria and identify mechanisms of action;

- Microencapsulation of *S. guianensis* essential oil prolonging its larvicidal activity in the control of *A. aegypti* and *C. quinquefasciatus* larvae;

- Identification of the mechanism of action of *C. alba* in the control of the Sars-CoV-2 virus.

- Use of *C. alba* extract to control the virus Sars-CoV-2 in vitro and the use of its root as an herbal medicine in humans.

3 LITERATURE REVIEW

3.1 Neotropical Plants

The neotropical region is characterized by several ecosystems, ranging from desert environments to humid forests. Among the neotropical biomes are the Amazon and Atlantic rainforests, Andean high-altitude grasslands – paramos, puna and jalca – Pampas, seasonally dry forests, savannas, cerrado and rocky grasslands (Hughes et al., 2013; Pennington & Lavin, 2016), Chaco (Meadow, 1993), deserts (Roig et al., 2009) and Pantanal (Pott et al., 2011).

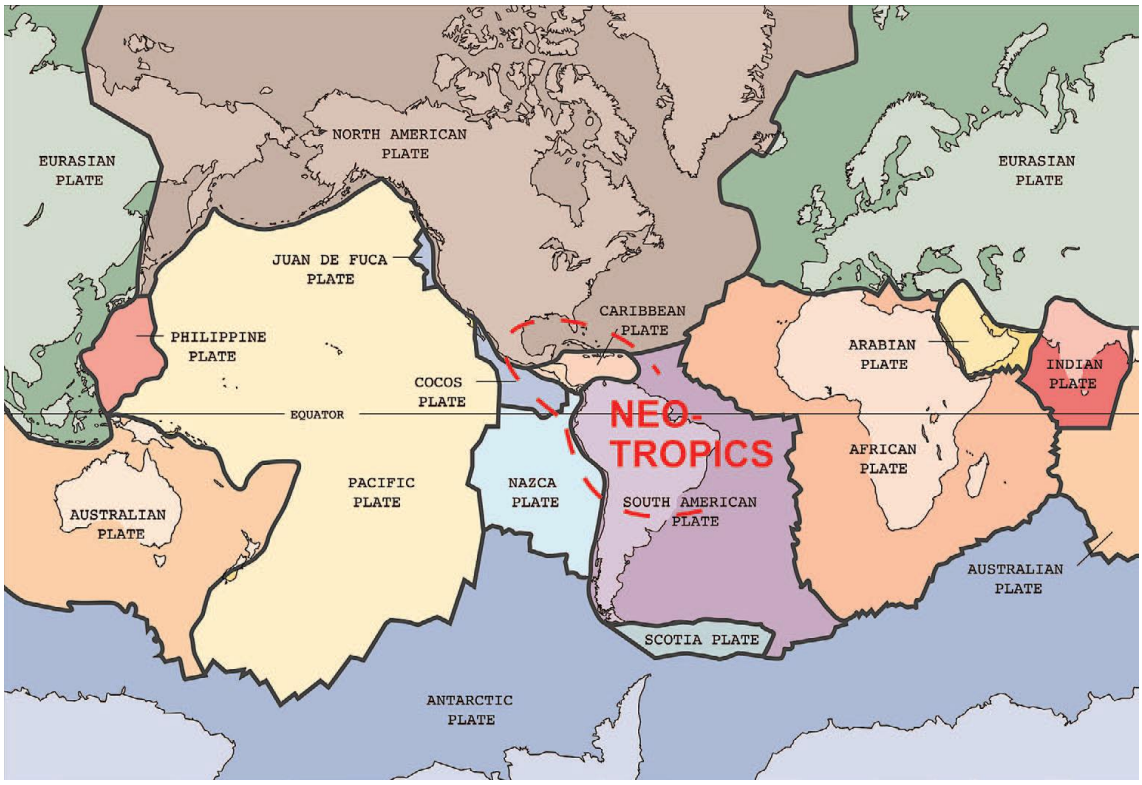

Figure 1 - The Neotropical region extends from central Mexico in northern to southern Brazil, thus occupying the North American, Caribbean and South American tectonic plates (Antonelli & Sanmartin, 2011).

Approximately 90,000-110,000 plant species are found in the Neotropics, which represents aproximately 37% of the world's species. This is perhaps more than tropical Africa (30,000–35,000 spp.), tropical Asia and Oceania combined (40,000–82,000 spp.) (Gentry, 1982; Govaerts, 2001). Within the Neotropics, as in any other region, plant species are not evenly distributed; in this region, there are two main patterns of plant distribution, the so-called “Amazon-centred” and “Andean-centred”. This immense biodiversity is drawing the attention of many researchers around the world in the search for alternatives for identifying new active molecules.

3.1.1 *Siparuna guianensis*

S. guianensis Aublet (Siparunaceae) was the first species of Siparuna described and illustrated by Aublet in the Histoire des plantes by La Guiana Françoise (1775). It occurs from Nicaragua, traversing northern South America to Paraguay, in high primary and secondary forest plains, with heights of 1200 meters, rarely 1400 meters (Renner & Hausner, 2005). The genus Siparuna occurs in most neotropical vegetation types at elevations between sea level and 3800 meters (Renner & Hausner, 2005). Based on this current distribution, Siparunaceae could be a large Western family of plants. The phylogenetically basal species of this family occur in all areas of the Amazonian lowlands and the protected areas of the Guianas,

suggesting that this group initially diversified after adapting to high altitudes, such as the elevations of the Andes.

Species *S. guianensis* presents itself as monoecious shrubs or arbors, that is, each plant has sexual organs of both sexes, between 5 and 9 meters in height, with smooth bark generally classified as gray, with small cylindrical and flat young branches. in the nodes (figure 2) (Valentini et al., 2010). According to Renner and Hausner (2005), this species has leaves classified as membranous, with smooth, opposite margins, with petioles varying between 0.5 and 1.5 centimeters in length and elongated to elliptical between 10 and 22 by 4 to 10 centimeters. Its base is defined as an obtuse, pointed apex. The lower surface has approximately 9 to 11 pairs of minor ribs slightly evident above.

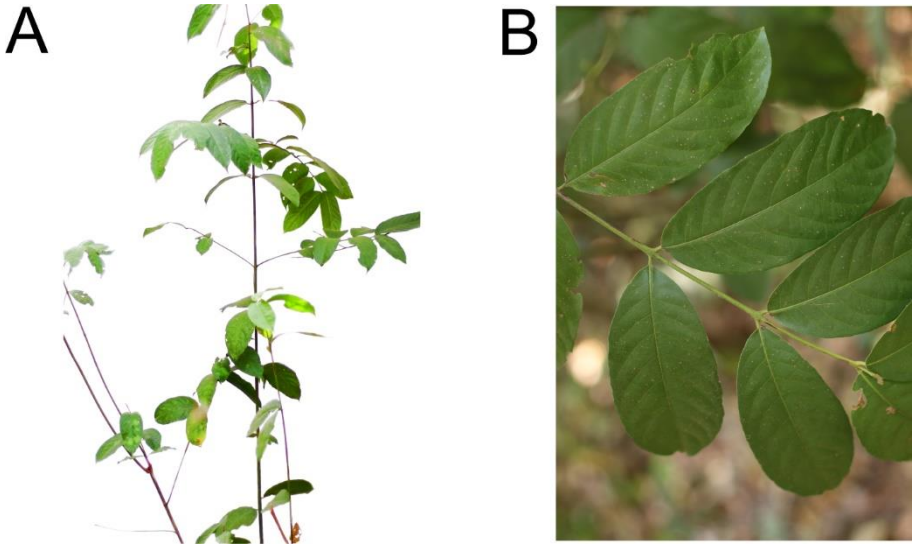

Figure 2 – *S. guianensis* (a) shrub with stem and leaves; (b) Sheets. (Font: Author)

In Brazil, it is located mainly in the Cerrado, where these plants are native and abundant. However, its reserves are threatened because they are not legally protected (Valentini et al., 2010). In Brazil, *S. guianensis* is used in folk medicine, and the leaves and flowers are considered calming, aromatic, antidepressant, diuretic, stimulant and have antifebrile properties (Valentini et al., 2010). In the studies, the essential oil *S. guianensis* was shown to be toxic to *Rhipicephalus microplus*, which parasitizes a variety of animal species (Diniz, 2014) and was also very efficient in controlling *A. aegypti* and *C. quinquefasciatus*, which are vector insects responsible for the transmission of several human diseases caused by protozoa (malaria), nematodes (lymphatic filariasis) and viruses (West Nile encephalitis, dengue, yellow fever and chikungunya fever) (Aguiar et al., 2015).

3.1.2 *Chiococca alba*

C. alba (L.) Hitchc. (Rubiaceae) was first described in 1777 by the Peruvian botanist José Pavon and the naturalist Hipolito Ruiz (Gazga, 2004); it has a neotropical distribution

(Steyermark 1974; Lorence 1999), and in Brazil, it was cited by Jung-Mendaçolli (1999) as an umbrophilous species found in several ecosystems such as the Amazon, Atlantic Forest and Cerrado, prevailing species ranging from semishrubs to trees (Bolzani et al., 2001).

The species *C. alba* is a shrub 2 to 3 meters tall, with thin branches, such as cross knots. The stem is cylindrical with a thin diameter and longitudinal grooves. The leaves are opposite and short, with a leathery, entire, elliptical limb with an obtuse base and a mucronate apex (Figure 3). Its flowers are tetramer, white-yellowish in color, the corolla is hypocrateriform, imbricate prefloration, glabrous with laces with greenish and turned edges. The isomeric stamens are free. Fillets slightly grow between themselves and the base of the corolla and are composed of hairs in the median region; linear anthers and inferior ovary, with two monosperm locules. The root is the most commonly used part of the plant and has an irregular shape, with a diameter ranging from 5 to 70 mm and 30 to 40 cm in length, covered by a brown bark with numerous transverse striations (Pires, 2020).

Figure 3 – *C. alba* (A) Leaves and flowers; (B) Root. (Font: Author)

Infusion of its roots has been used in traditional Brazilian medicine for the treatment of various diseases due to its anti-rheumatic, diuretic, anti-inflammatory, antiviral, and anti-snake bite properties (Mors et al., 2000) and its ability to cure intermittent fever (Antonelli & Sanmartin 2011). C. alba was listed in the first Brazilian pharmacopeia, as well as in several European pharmacopeias (Schapoval et al., 1983). A prescribed herbal remedy for rheumatism containing the ethanol extract of the roots of *C. alba* together with an extract of *Polypodium lepidopterous* was on the Brazilian pharmaceutical market until the nineties (Borges et al, 2013).

3.2 Bacteria resistance

Bacteria are widely distributed in the environment and are a growing cause of serious infections in hospitals, where they mainly affect patients immunocompromised by underlying diseases or by medical treatments and surgical procedures. Many species are known for their resistance to all classes of antimicrobials and for the ease with which they can acquire new resistance mechanisms (Enoch et al., 2007).

Several factors contribute to bacterial resistance, including inherent adaptability and horizontal gene transfer, as well as the ability of bacteria to mutate. Additional factors also promote resistance in bacteria, including misuse and overuse of antibiotics, inappropriate dosing, spillage of antibiotics into the environment, and growth-promoting antibiotics used in the livestock industry. These factors cumulatively result in a significant reduction in the antibiotic resistance-free time interval (Valdes-Pena et al., 2021).

3.2.1 *Escherichia coli*

E. coli is a bacterium of the Enterobacteriaceae family, of the coliform group, composed of gram-negative bacteria, straight rods, facultative anaerobes and negative oxidase and ferment sugars. Among them are lactose fermenting bacteria belonging to the coliform group, which are differentiated by the term's total coliforms and thermotolerant coliforms (Oliveira, 2015). *E. coli* pathogenicity is manifested by a multifactorial and complex mechanism that involves several factors that vary according to the serotype, presenting, several enteric and extraintestinal pathologies caused by some *E. coli* serotypes (Rocha, 2008).

In recent years, the indiscriminate use of antibiotics has promoted an increase in resistant *E. coli* strains. According to Balkhy et al. (2015) there is increased resistance of *E. coli* to antibiotics in low- and middle-income countries, whose health systems are precarious and lack the tools to quickly diagnose the numerous neglected infectious diseases. Thus, greater efforts must be made especially in these locations to contain the spread of multi-antibiotic-resistant *E. coli*, especially with their moderate antibiotic prescribing policies.

Antibiotic resistance it is related to increased poverty, and antibiotic misuse is associated with the transport of resistant *E. coli* from healthy children into community settings across the world (Malik & Bhattacharyya, 2019). In high-income countries, strict antibiotic prescribing policies are in place to reverse the course of antibiotic resistance (Sanches et al., 2016).

3.2.2 *Staphylococcus aureus*

S. aureus is a gram-positive bacterium belonging to the Staphylococcus genus of the Staphylococcaceae family. They are usually grouped in the form of coconuts with the appearance of bunches of grapes. They are immobile, nonsporogenic, catalase-positive and generally oxidize negative. As they are chemoorganotrophic, they have respiratory and fermentative carbohydrate metabolism. They are susceptible to lysis by lysostaphin and resistant to lysozyme. Predominantly associated with the skin, glands and mucous membranes of warm-blooded animals (Silva, et al., 2017).

They are part of the human microbiota and can cause illnesses ranging from a simple infection, such as pimples and boils, to more serious ones, such as pneumonia, meningitis, endocarditis, toxic shock syndrome and septicemia. In humans, they are the bacteria most frequently found in the nasal mucosa, from which they contaminate the hands, playing an important role in the spread of infections through food (Almeida et al., 2016). Their great interaction and adaptation to humans, animals and the environment and their easy acquisition of genes responsible for enhancing their pathogenicity factors make them a major public health problem (Souza et al., 2017).

The discovery of the resistance of this bacterium to antibiotics has promoted several studies, as they are classified as pathogens causing diseases linked to animals (Barrera-Rivas et al., 2017) and humans (Alegre et al., 2016), and the search for options to control its development through alternative methods has been widely discussed.

3.2.3 *Pseudomonas aeruginosa*

P. aeruginosa is a gram-negative bacillus, presenting a monotic, nonspore-forming and strictly aerobic flagellum. Furthermore, it can appear as an isolated cell, in pairs or short chains (Aedekerk et al., 2002; Aeschlimann et al., 2003). An important feature of this group of microorganisms is the ability to produce fluorescent pigments such as pyoverdine, pyocyanin, pyorubin and pyomelanin. The production of these pigments can help identify species of this bacterial genus (Andrade et al., 2003). *P. aeruginosa* can be considered the most virulent species of this family due to the factors that characterize it, as well as the ability to adhere to host cells through the fimbriae, production of polysaccharides (alginate), extracellular toxins and the presence of lipopolysaccharides in the cell wall (Arakawa et al., 2000).

This bacterium is known to cause acute infections characterized by the production of toxins and chronic infections by the production of a thick layer of biofilm. Its pathogenesis is directly related to the host's condition, affecting mainly burn patients, cystic fibrosis patients and ICU patients whose immune system is weakened (Musbah et al, 2015). Infections caused by *P. aeruginosa* are difficult to treat, as these microorganisms have high levels of resistance

to various antimicrobials and express several virulence factors that contribute to the establishment of persistent infections (Tanya et al, 2009).

The multidrug resistance characteristics of this microorganism are associated with the presence of enzymes and mutations that can occur alone or simultaneously (Papoff et al., 2012); however, for the control and elimination of the infection, the drugs of choice are those belonging to the β-lactams. groups, aminoglycosides, polymyxins and fluoroquinolones (Blair et al., 2009).

3.2.4 *Streptococcus pyogenes*

S. pyogenes are gram-positive cocci grouped in pairs or chains. The cell wall composition is similar to that of other gram-positive bacteria, being formed by glycopeptides, in which several carbohydrates, teichoic acids, lipoproteins and surface antigenic proteins are inserted. It is believed that *S. pyogenes* is a strict human pathogen with a narrow ecological niche, which prevents researchers from studying the potential zoonotic aspect (Koneman et al., 2001).

Among the diseases caused by these streptococci are postpartum infections, bacteremia, necrotizing fasciitis, cellulitis, myositis, puerperal sepsis, meningitis, pneumonia, scarlet fever, toxic shock syndrome and immunologically mediated nonsuppurative sequelae (rheumatic fever and poststreptococcal glomerulonephritis). Puerperal endometritis can also be caused by group A streptococci, and although the number of cases has declined in developed countries over the past few decades, severe outbreaks still occur (Walker et al., 2014).

S. pyogenes manifested itself in recent decades causing severe invasive infections with high morbidity and mortality worldwide. It is estimated that there are approximately 663,000 new cases of invasive S. pyogenes infections annually and 163,000 associated deaths worldwide (Kalgo et al., 2018). This was due to the empirical use of antibiotics, which led to the emergence of resistant bacterial strains that constitute a major challenge for clinicians in limiting drug choices (Shulman et al., 2012).

3.4 Disease vector insects

The scientific community has shown interest in studying insects for a long time. From the first half of the nineteenth century, entomology was individualized as a science, when the Entomological Societies of Paris (1832), London (1833) and Germany (1887) emerged. At that time, doctors, parasitologists, bacteriologists, zoologists, veterinarians and botanists were interested in studying possible transmitters of infectious and parasitic diseases (Machado, 1987).

Insects arouse several interests with ecological, agricultural and public health importance. As vectors of human diseases, they directly affect people and domestic animals, mainly mosquitoes (malaria, dengue, filariasis, elephantiasis and yellow fever), lice (typhus and recurrent fever), fleas (bubonic plague), and a legion of house flies, responsible for typhoid fever and dysentery (Edler, 2011). Mosquitoes are the most important insects for public health due to their vectorial capacity and competence to transmit pathogens, including arboviruses, bacteria and parasites (Segura et al., 2021).

3.3.1 *Aedes aegypti*

A. aegypti Linnaeus, 1762 (Diptera: Culicidae), is responsible for epidemics caused by arboviruses such as dengue, chikungunya and Zika, which have spread widely around the world due to several factors related to human behavior (Kyeongah et al., 2016; Ferreira-de-Brito et al., 2016; Weaver & Lecuit, 2015).

It is estimated that approximately 60 million people worldwide are affected by the dengue virus each year, resulting in approximately 10,000 deaths (Bhatt et al. 2013; Stanaway et al., 2016). The number of dengue cases in Brazil has been increasing considerably in recent years; in 2020, there was an increase of 70% in cases in the first three months of the year compared to the previous year, with the midwestern region being responsible for most of the cases (Brazil, 2020).

It is noteworthy that there are still no vaccines that can prevent these diseases, and therefore the best strategy to fight these diseases is the control of *A. aegypti* larvae and their adult populations, for which the application of synthetic insecticides is still the most commonly used method in the world (Aguiar et al., 2015).

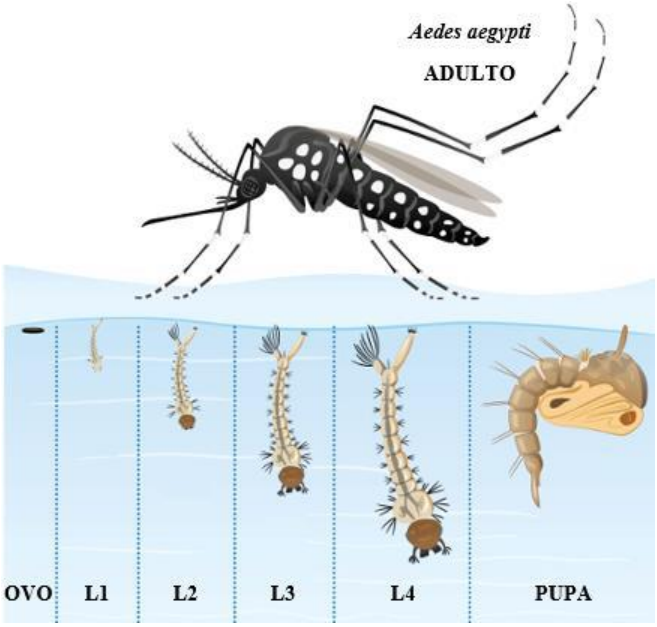

Figure 4 - Developmental cycle of *A. aegypti* (adult, eggs, larval instars and pupa). (Font: Santos, 2018).

The continuous application of insecticides against *A. aegypti* eggs and larvae has resulted in the selection of resistant insects. Resistance mechanisms in arthropod vectors are caused by behavioral or physiological changes. Physiological responses in the form of target site modifications alone may be sufficient to confer pyrethroid resistance in *A. aegypti* (Scott, 2019). Metabolic resistance mechanisms are also very well investigated in *A. aegypti*, aiding in the detoxification of xenobiotics through increased expression or gene alterations (Hemingway et al., 2004).

In this context, there is a need for alternative methods, especially those based on natural resources, and the insecticide must be sustainable, ecologically correct, effective, have low toxicity to mammals and must not significantly modify the characteristics of the water (Dias & Moraes, 2014), highlighting the importance of adding toxicity studies to nontarget organisms for the implementation of alternative vector controls.

3.3.2 *Culex quinquefasciatus*

C. quinquefasciatus Say, 1823 (Diptera: Culicidae) is considered a vector of the roundworm Wuchereria bancrofti (Cobbold, 1877), which causes lymphatic filariasis disease, commonly known as elephantiasis. In 2013, the World Health Organization released a document explaining the importance of vector control, as this insect parasite is an endemic vector in many tropical regions (Anju et al. 2020).

Approximately 120 million infected individuals in 81 countries and more than 1 billion people are at risk of infection (WHO, 2009), and in Brazil the endemic area is located mainly in the municipalities of Recife, Jaboatão dos Guararapes, Olinda and Paulista , belonging to the Metropolitan Region of Recife, in the state of Pernambuco (Brazil, 2017).

Several studies report the resistance of *C. quinquefasciatus* larvae to insecticides. This resistance is mainly due to two molecular mechanisms: mutations in the target site - changing the insecticide target molecule and metabolic resistance - involving increased activity of detoxification enzymes (Hemingway et al., 2004; Pocquet et al., 2013; Talipouo et al., 2021).

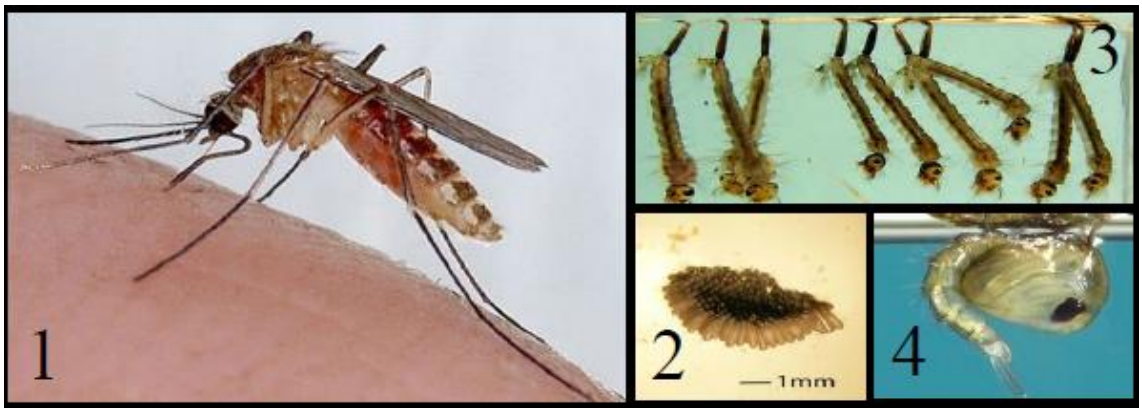

Figure 5 - Developmental stages of *C. quinquefasciatus*: 1. Adult mosquito; 2 eggs; 3. Larvae; 4. Pupa. (Font: Santos, 2018)

The use of plants has been studied as a way to control the spread of these insects, since the use of their compounds has shown positive results in larval mortality (Santos, 2018). An alternative system used to control *C. quinquefasciatus* larvae was nanoemulsions, where the preparation of nanoemulsions of natural oils has been shown to be a promising technology for the control of these insects (Anjali et al., 2012).

3.4 COVID-19

3.4.1 Sars-Cov-2

In late 2019, a disease known as new Coronavirus (COVID-19) caused by SARS-CoV-2 was reported in Wuhan, the sprawling capital of Hubei Province, with approximately 11 million people in the central region of the People's Republic. of China (Zhu et al., 2020). It subsequently expanded across the world, affecting more than 26 countries (Aanouz et al., 2020). On January 30, 2020, the World Health Organization (WHO) proclaimed international concern with the public health emergency and on March 11, 2020, the virus was confirmed as a pandemic (Rosales-Mendoza et al., 2020). The WHO reported on 15 April 2020 that there were 1,914,916 confirmed cases of infection present in 213 countries, areas or territories worldwide, along with 123,010 deaths; as of October 18, 2020, there were more than 40 million confirmed cases of infection worldwide, along with 1.1 million deaths (Rosales-Mendoza et al., 2020).

The COVID-19 outbreak is the sixth public health emergency of global concern, after H1N1 (2009), polio (2014), Ebola (2014 - West Africa), Zika (2016) and Ebola (2019 - Democratic Republic of Congo) (Dey et al., 2020. Similar to MERS and SARS, there are no distinct clinical features of COVID-19 and the symptoms significantly overlap with other severe acute respiratory infections (Chen et al., 2020; Huang et al., 2020). Clinical characterization protocols are now being collected from patients around the world to better define the disease,

in terms of its natural history, mode of transmission, clinical profiles, management and specific risk factors to prevent or overcome harmful effects. of the disease (Arabi et al., 2020).

COVID-19 replicates efficiently in the upper respiratory tract, and infected patients generate a large amount of viruses contributing to the spread of the infection (Chan et al., 2020). Nonpharmaceutical interventions are essential for the management of COVID-19, along with available licensed vaccines or even antivirals for coronaviruses (Heymann & Shindo, 2020). Based on clinical observations, it is clear that the proportion of individuals infected with COVID-19 who remain asymptomatic throughout the infection has not yet been definitively evaluated. Even these asymptomatic patients can potentially be a source of infection.

In this sense, several studies are being carried out to try to identify possible drugs with antiviral activity using in silico tools (Cava et al., 2020; Russo et al., 2020) and in vitro assays (Vashist, 2020; Khan et al., 2021) and some studies are already taking place with clinical trials in advanced stages (Silveira et al., 2021). Herbal treatments may be an alternative in the discovery of new compounds in the treatment of COVID-19 (Bhuiyan et al., 2020).

3.5 Applications of plant compounds

In recent years, interest in the use of plant compounds such as essential oils, plant extracts and their components in the most diverse applications has been growing. The essential oil of *Ocimum Mild*, for example, contains some sesquiterpenoids, which include germacrene-D (29.2%) and germacrene-B (14.0%). This essential oil showed good antimicrobial activity against four gram-positive bacteria (*S. aureus*, *S. epidermidis*, *S. mutans* and *S. viridans*) and four gram-negative bacteria (*P. aeruginosa*, *E. cloacae*, *K. pneumoniae* and *E*. *coli*) (Runyoro et al., 2010).

Compounds such as Germacrene-B, Germacrene-D and myrcene were also identified when essential oils from *Helosciadium nodiflorum*, *Pimpinella anisum*, *Smyrnium olusatrum* and *Trachyspermum ammi* were used synergistically in the control of *C. quinquefasciatus* (Benelli et al., 2017). The essential oil of *Clausena excavata*, which shows good larvicidal activity against *A. aegypti*, contains germacrene B and D and also β-myrcene (Cheng et al., 2009).

Among the plant compounds used as antiviral agents, the methanol extract of *Erythrina speciosa* leaves, which was subsequently partitioned with ethyl acetate, demonstrated inhibition of HSV-1 (herpes simplex virus), demonstrating EC_{50} and SI values of 94 μg/ml and 2.65, respectively. This extract contains the molecule vitexin as its main component, which has been shown to be a potential antiviral candidate (Fahmy et al., 2020).

Naringin is also a plant compound classified as a flavonoid with antiviral, anticancer and hepatoprotective effects. Flavonoids are a class of natural compounds ubiquitous in the

plant kingdom known for their phytopharmacological capacity, and naringin is found in plants such as *Exocarpium citri*, *Passiflora edulis* and *Chelidonium Majus* (Jung et al., 2003).

REFERENCES

Aanouz I, Belhassan A, El-Khatabi K, Lakhlifi T, El-Ldrissi M, Bouachrine M. Moroccan Medicinal plants as inhibitors against SARS-CoV-2 main protease: Computational investigations. J Biomol Struct Dyn. 2021 May;39(8):2971-2979. doi: 10.1080/07391102.2020.1758790

Aedekerk, S. *et al* Characterization of a new efflux pump, MexGHI-OpmD, from *Pseudomonas aeruginosa*that confers resistance to vanadium. *Microbiology*, v. 148, n. 8, p. 2371-81, 2002.

Aeschlimann, J. R. The role of multidrug efflux pumps in the antibiotic resistance of *Pseudomonas aeruginosa*and other Gram-negative bacteria. *Pharmacotherapy*, v. 23, n. 7, p. 916-24, 2003.

Aguiar, R.W.S.; Dos Santos, S.F.; Da Silva Morgado, F.; Ascencio, S.D.; De Mendonça lopes, M.; Viana, K.F. Insecticidal and Repellent Activity of *Siparuna guianensis* Aubl. (Negramina) against *Aedes aegypti* and *Culex quinquefasciatus*. PLoS ONE 10(2): e0116765. doi:10.1371/journal. 2015.

Alegre, M. L., chen, L., david, M. Z., Bartman, C., Susan B.V., Kumar, n., Chong, A. S., & Daum, R. S. (2016). Impact of *Staphylococcus aureus* USA300 Colonization and Skin Infections on Systemic Immune Responses in Humans. *The Journal of Immunology*, 197(4), 1118-1126. https://doi.org/10.4049/jimmunol.1600549

Aliaga-Samanez A, Cobos-Mayo M, Real R, Segura M, Romero D, Fa JE, et al. (2021) Biogeografia dinâmica mundial da dengue zoonótica e antroponótica. PLoS Negl Trop Dis 15 (6): e0009496. https://doi.org/10.1371/journal.pntd.0009496

Almeida, M.S.C., Mendonça, R.L., Freitas M.Z.C., & Vandesmet L.C. (2016). Staphylococcus aureus. Scientific exhibition in biomedicine, 1 (01).

Andrade, S. S. *et al* Increasing prevalence of antimicrobial resistance among *Pseudomonas aeruginosa*isolates in Latin American medical centres: 5 year report of the SENTRY Antimicrobial Surveillance Program (1997-2001). *J Antimicrob Chemother*, v. 52, n. 1, p. 140-1, 2003.

Anjali, C.H. ; Sharma, Y.; Mukherjee, A.; Chandrasekaran, N. .Neem oil **(***Azadirachta indica*) nanoemulsion a potent larvicidal agent against *Culex quinquefasciatus.* Pest Manag. Sci., 68 (2012), pp. 158-163, 10.1002/ps.2233

Antonelli A & Sanmartin I (2011) Why are there so many plant species in the Neotropics? Taxon 60: 403-414.

Arabi, Y.M., Murthy, S. & Webb, S. COVID-19: a novel coronavirus and a novel challenge for critical care. *Intensive Care Med* 46, 833–836 (2020). https://doi.org/10.1007/s00134-020-05955-1

Arakawa, Y. *et al* Convenient test for screening metallo ß-lactamase-producing Gram-negative bacteria by using thiol compounds. *J Clin Microbiol*, v. 38, n. 1, p. 40-3, 2000.

Astolfi Filho, S.; Nunes-Silva, C.G.; Bigi, M.F.M.A. Bioprospecting and biotechnology. Strategic Partnerships. Brasília-DF 2014, 19, 45–80.

Balkhy, H. H. *et al.* Antimicrobial resistance: One world, one fight! *Antimicrob. Resist. Infect. Control* 4 (2015).

Barrera-Rivas, C., Valle-Hurtado, N., González-Lugo, G., Baizabal-Aguirre, V., Bravo-Patiño, A., Cajero-Juárez, M., & Valdez-Alarcón, J. (2017). Bacteriophage Therapy: An Alternative for the Treatment of *Staphylococcus aureus* Infections in Animals and Animal Models. Frontiers in *Staphylococcus aureus*. 10.5772/65761

Benelli, G. ; Pavela, R.; Iannarelli, R. ; Petrelli, R. ; Cappellacci, K.; Cianfaglione,G.; Afsharf, F.H. ; Nicoletti, M. ; Canale, A. ; Maggi, F. Synergized mixtures of Apiaceae essential oils and related plant-borne compounds: larvicidal effectiveness on the filariasis vector *Culex quinquefasciatus* Say. Ind. Crop. Prod., 96 (2017), pp. 186-195, 10.1016/j.indcrop.2016.11.059

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., 2013. The global distribution and burden of dengue. Nature. 496 (7446): 504–507.

Bhuiyan, R. ; Howlader, S. ; Raihan, T.; Hasan, M. Plants metabolites: possibility ofnatural therapeutics against the COVID-19 pandemic, Front. Med. 7 (2020) 444,https://doi.org/10.3389/fmed.2020.00444

Blair, J. *et al* Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. *Current Opinion in Microbiology*, v. 12, n. 5, p. 512-9, 2009.

Bolzani, V. S.; Yong, M. C. M.; Furlan, M.; Cavalheiro, A. J.; Araújo, A. R.; Silva, D. H. S. & Lopes, M. N. Secondary Metabolites from Brazilian Rubiaceae Plant Species: Chemotaxonomical and biological significance. Recent Research Development in Phytochemistry, 5: 19-31, 2001.

Borges, R. M.; Valenca, S. S.; Lopes, A. A.; Barbi, N. S; Silva, A. J. R. Saponins from the roots of *Chiococca alba* and their in vitro anti-inflammatory activity. Phytochemistry Letters, v.6(1), p. 96-100, 2013.

Brazil. Ministry of Health (2020). Monitoring of cases of urban arboviruses transmitted by Aedes Aegypti (dengue, chikungunya and zika), Epidemiological weeks 1 to 11, 2020. Epidemiological Bulletin. 51(12).

Cava, C.; Bertoli, G.; Castiglioni, I. In Silico Discovery of Candidate Drugs against Covid-19. *Viruses* 2020, *12*, 404. https://doi.org/10.3390/v12040404

Chan et al., Engineering human ACE2 to optimize binding to the spike protein of SARS coronavirus 2. Science 369, 1261–1265 (2020)

Chen N, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020;395:507–513. doi: 10.1016/s0140-6736(20)30211-7.

Cheng, S.S.; Chang, H.T. ; Lin, C.Y. ; Chen, P.S.; Huang, C.G. ; Chen, W.J. ; Chang, S.T. Insecticidal activities of leaf and twig essential oils from *Clausena excavata* against *Aedes aegypti* and *Aedes albopictus* larvae. Pest Manag. Sci., 65 (2009), pp. 339-343. Doi:10.1002/ps.1693

Conservancy TN (2005) Evaluación ecoregional del Gran Chaco Americano. Wildlife Conservation SocietyBolivia, Buenos Aires. 24p.

Cruz, J. Cainca: Estudo Botânico, Pharmacognostico e Chimico**.** Boletim do Museu Nacional (1930) pp. 97–116.

Dey, S.K. ; Rahman, M.M.; Siddiqi, U.R. ; Howlader, A. Analyzing the epidemiological outbreak of COVID-19: a visual exploratory data analysis approach. J. Med. Virol., 92 (6) (2020), pp. 632-638

Dias, C. N., & Moraes, D. F. C. (2014). Essential oils and their compounds as Aedes aegypti L.(Diptera: Culicidae) larvicides.Parasitology research,113(2), 565-592.

Diniz, J.A. *Siparuna guianensis* Aublet como nova fonte de α-bisabolol para o controle de *Rhipicephalus microplus*. Dissertação de Mestrado, 94p universidade Federal de Viçosa, Minas Gerais, Brasil. 2014.

Edler, F. A medicina no Brasil Imperial: clima, parasitas e patologia trópica. Rio de Janeiro: Editora Fiocruz, 2011.

Enoch, D.A., Birkett, C.I. and Ludlam, H.A. (2007) Non Fermentative Gram-Negative Bacteria. International Journal of Antimicrobial Agents, 3, 33-41.

Fahmy, NM, Al-Sayed, E, Moghannem, S, Azam, F, El-Shazly, M, Singab, AN. Breaking down the barriers to a natural antiviral agent: antiviral activity and molecular docking of erythrina speciosa extract, fractions, and the major compound. Chem Biodivers. 2020;17(2):e1900511.

Ferreira-de-brito, A., Ribeiro, I.P., Miranda, R.M., Fernandes, R.S., Campos, S.S., Silva, K.A.B., Castro, M.G., Bonaldo, M.C., Brasil, P., Lourenço-de-oliveira, R., 2016. First detection of natural infection of Aedes aegypti with Zika virus in Brazil and throughout South America. Mem. Inst. Oswaldo Cruz. 111: 655–658. https://doi:10.1590/0074-02760160332

Gentry, A.H. 1982. Neotropical floristic diversity: Phytogeographical connections between Central and South America, Pleistocene climatic fluctuations, or an accident of the Andean orogeny? *Ann.Missouri Bot. Gard*. 69: 557–593.

Govaerts, R. 2001. How many species of seed plants are there? *Taxon* 50: 1085–1090.

Hemingway, J., Hawkes, N. J., McCarroll, L. & Ranson, H. The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem. Mol. Biol.* 34, 653–665 (2004).

Heymann, D. L., & Shindo, N. (2020). COVID-19: what is next for public health? The Lancet, 395, 542–545.

Huang C, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020;395:497–506. doi: 10.1016/s0140-6736(20)30183-

Hughes CE, Pennington RT & Antonelli A (2013) Neotropical plant evolution: assembling the big picture. Botanical Journal of the Linnean Society 171: 1-18.

Jung, U., Kim, H., Lee, J., Lee, M., Kim, H., Park, E., et al. (2003). Naringin supplementation lowers plasma lipids and enhances erythrocyte antioxidant enzyme activities in hypercholesterolemic subjects. Clinical Nutrition, 22(6), 561–568

Jung-Mendaçolli, S.L. 1999. Flora fanerogâmica da Ilha do Cardoso (São Paulo, Brasil) Rubiaceae. Pp. 45-136. In: M.M.R.F. Melo *et al.* (eds.). Flora Fanerogâmica da Ilha do Cardoso v. 6. Instituto de Botânica, São Paulo.

Kalgo, H.M.; Jasni, A.S.; Hadi, S.R.A.; Umar, N.H. Extremely low prevalence of erythromycin-resistant *Streptococcus pyogenes* isolates and their molecular characteristics by M Protein gene and multilocus sequence typing methods. Jundishapur J. Microbiol., 11 (2018), p. e12779

Khan, T., Khan, M. A., Karam, K., Ullah, N., Mashwani, Z. U., & Nadhman, A. (2021). Plant *in vitro* Culture Technologies; A Promise Into Factories of Secondary Metabolites Against COVID-19. *Frontiers in plant science*, *12*, 610194. https://doi.org/10.3389/fpls.2021.610194

Koneman, E. W.; Allen, S.D.; Janda, W. M.; Schreckenberger, P.C.; Winn, W.C. Diagnóstico Microbiológico. 5ª edição. 2001. MEDSI Editora Médica e Científica Ltda. Rio de Janeiro – RJ, p. 589-614.

Kyeongah, N., Kenji, M., Yuichiro, M., Yohei, Y., Ryo, K., Hiroshi, N., 2016. Estimating risks of importation and local transmission of Zika virus infection. PeerJ 4 (3):1904.

Lima-Camara, T. N. Emerging arboviruses and public health challenges in Brazil. Revista de Saúde Pública, v. 50, 2016.

Loannidis, J. P.A.; Axfors, C, Contopoulos-Ioannidis, D.G. (2021).Second versus first wave of COVID-19 deaths: Shifts in age distribution and in nursing home fatalities. Environmental Research. 195. 10856. https://doi.org/10.1016/j.envres.2021.110856.

Lorence, D.H. 1999. A nomenclator of Mexican and Central American Rubiaceae Missouri Botanical Garden Press, St. Louis.

Machado, P. A. O Homem e os insetos: passado, presente e futuro. Rev. Saúde Pública. 21(6): 474-479. 1987

Malik, B. & Bhattacharyya, S. Antibiotic drug-resistance as a complex system driven by socio-economic growth and antibiotic misuse. *Sci. Rep.* 9, 9788 (2019).

Mors, W.B. Mors; Rizzini, C.T.; Pereira, N.A. (2000). Medicinal Plants of Brazil Reference Publications, Michigan pp. 297.

Musbah, A.S.; Omar, R.; Ismail, S.; Yusof, M.Y. Inhibition of quorum sensing-controlled virulence factors and biofilm formation in Pseudomonas aeruginosa by culture extract from naovel bacterial species of paenibacillus using a rat Model of Chronic Lung Infection. International Journal of Bacteriology Vol. 15. 2015.

Negri, G., Santi, D., Tabach, R. 2012. Chemical composition of hydroethanolic extracts from *Siparuna guianensis*, medicinal plant used as anxiolytics in Amazon region. Braz. J. Pharmacog. 5:1024- 1034.

Oliveira, M. A.; Takamura, A. E.; Arias vigoya, A. A., Araújo, F.E. Enterobacteriaceae: bactérias intestinais de organismos aquáticos, um risco à saúde pública – revisão de literatura. Revista Científica de Medicina Veterinária - Ano XIII, n 25, 2015

Oliveira, M. S., Cruz, J. N., Mitre, G. P., Costa, W. A., Kataoka, M. S. S., Silva, S. G., Alves, A. C. B. A., Pinheiro, J. J. V., Silva, S. E. M., Menezes, S. A. F., Menezes, T. O. A., Neto, A. M. J. C., Junior, R. N. C., 2019. Antimicrobial, cytotoxic activity of the *Syzygium aromaticum* essential oil, molecular docking and dynamics molecular studies of its major chemical constituent. J. Comp. Theor. Nanosci. 16(2), 355-364. https://doi.org/10.1166/jctn.2019.8108

Papoff, P, Ceccarelli, G., d'Ettorre, G., Cerasaro, C., Caresta, E., Midulla, F. Gut Microbial Translocation in Critically Children and Effects of Supplementation with Pre- and Pro Biotics. International Journal of Microbiology. 2012.

Pennington RT & Lavin M (2016) The contrasting nature of woody plant species in different Neotropical forest biomes reflects differences in ecological stability. New Phytologist 210: 25-37.

Pereira, A.A. et al. Chemical characterization and inhibitory effect of essential oils on the growth of Staphylococcus aureus and Escherichia coli. Science and Agrotechnology. 2008, v. 32, n. 3 pp. 887-893. doi.org/10.1590/S1413-70542008000300028

Pocquet, N. *et al.* Multiple insecticide resistances in the disease vector Culex p. quinquefasciatus from Western Indian Ocean. *PLoS ONE* 8, 77855 (2013).

Pott A, Oliveira AKM, Damasceno GA & Silva JSV (2011) Plant diversity of the Pantanal wetland. Brazilian Journal of Biology 71: 265-273.

Prado DE & Gibbs PE (1993) Patterns of Species Distributions in the dry seasonal forests of South- America. Annals of the Missouri Botanical Garden 80: 902-927.

Raghubanshi, B.R.; Sagili, K.D.; Han, W.W.; Shakya, H.; Shrestha, P.; Satyanarayana, S.; Karki, B.M.S. Antimicrobial Resistance among Neonates with Bacterial Sepsis and Their Clinical Outcomes in a Tertiary Hospital in Kathmandu Valley, Nepal. *Trop. Med. Infect. Dis.* 2021, *6*, 56. https://doi.org/10.3390/tropicalmed6020056

Rahman, A. A. A., Mohamed, S. M., Samy, E. M., Sayed, M. .A., Youssef, E. M. K, Hetta, H.F., 2018. Novel Formulation and Clinical Evaluation of Levofloxacin Hemihydrategel for the Treatment of Impetigo. J. Am. Sci. 14(10), 47-57. https://doi:10.7537/marsjas141018.06.

Renner, S. S.; G. HAUSNER. Monograph of Siparunaceae. Flora Neotropical, v. 95, 256p., 2005.

Riva , L. , Yuan , S. , Yin , X. , Martin-Sancho , L. , Matsunaga , N. , Burgstaller , S. , Chan , JF et al. (2020). Uma Pesquisa de Reposicionamento de Medicamentos em Grande Escala para os antivirais SARS-CoV-2. bioRxiv. doi: https://doi.org/10.1101/2020.04.16.044016

Rocha, SL Detection of virulence factors of,*Escherichia coli* fertilizers from poultry farms in RS using MultiplexPCR. Masters dissertation. 2008. 68 f. University of Rio Grande do Sul.

Roig FA, Roig-Junent S & Corbalan V (2009) Biogeography of the Monte Desert. Journal of Arid Environments 73: 164-172.

Rosales-Mendoza, S., Márquez-Escobar, V. A., González-Ortega, O., Nieto-Gómez, R., Arévalo-Villalobos, J. I. (2020). What Does Plant-Based Vaccine Technology Offer to the Fight against COVID-19? *Vaccines* 8, 183. doi: 10.3390/vaccines8020183

Runyoro, D., Ngassapa, O., Vagionas, K., Aligiannis, N., Graikou, K., & Chinou, I., 2010. Chemical composition and antimicrobial activity of the essential oils of four *Ocimum* species growing in Tanzania. Food Chem. 119(1), 311–316. https://doi:10.1016/j.foodchem.2009.06.028

Russo, G., Pennisi, M., Fichera, E. *et al.* In silico trial to test COVID-19 candidate vaccines: a case study with UISS platform. *BMC Bioinformatics* 21, 527 (2020). https://doi.org/10.1186/s12859-020-03872-0

Sanchez, G. V., Fleming-Dutra, K. E., Roberts, R. M. & Hicks, L. A. Core elements of outpatient antibiotic stewardship. *MMWR Recomm. Rep.* 65, 1–12 (2016).

Santos, E.L.V.D.S.Larvicidal activity of the essential oil nanoemulsion of Ocimum basilicum Linn (Lamiales: Lamiacea) on Aedes aegypti Linnaeus and Culex quinquefasciatus Say Diptera: Culicidae. Masters dissertation. 63p. Federal University of Amapá. 2018.

Schapoval, E.E.S.; Schenkel, E.P.; Chaves, C.M.G.; Zanata, A. Anais do 2° Simpósio de Produtos Naturais, João Pessoa (1983)

Scott, J. G. Life and death at the voltage-sensitive sodium channel: Evolution in response to insecticide use. *Annu. Rev. Entomol.* 64, 243–257 (2019).

Segura, N. A.; Muñoz, A. L.; Losada-Barragán, M.; Torres, O.; Rodríguez, A. K.; Rangel, H.; Bello, F. Minireview: Epidemiological impact of arboviral diseases in Latin American countries, arbovirus-vector interactions, and control strategies. Pathogens and Disease. 2021. ftab043. https://doi.org/10.1093/femspd/ftab043

Shulman, S.T.; Bisno, A.L.; Clegg, H.W.; Gerber, M.A. Clinical practice guideline for the diagnosis and management of group A streptococcal pharyngitis: 2012 update by the Infectious Diseases Society of America Clin. Infect. Dis., 55 (2012), pp. 1279-1282

Silva, M.P, Cavalli, D.R, Oliveira, T.C.R.M. Avaliação do padrão coliformes a 45ºC e comparação da eficiência das técnicas dos tubos múltiplos e Petrifilm EC na detecção de coliformes totais e Escherichia coli em alimentos. Ciência Tecnologia de Alimentos, 26(2): 352-359, 2006.

Silva, N., Junqueira, A.C.V, Silveira, A.F.N, Taniwaki, H.M., Gomes, R.A.R, & Okazaki, M.M. (2017). Manual of methods for microbiological analysis of food and water (5th ed.). Blucher.

Silveira, M.A.D.; De Jong, D.; Berretta, A.A.; dos Santos Galvão, E.B.; Ribeiro, J.C.; Cerqueira-Silva, T.; Amorim, T.C.; daConceição, L.F.M.R.; Gomes, M.M.D.; Teixeira, M.B.; et al. Efficacy of Brazilian Green Propolis (EPP-AF®) as an adjunct treatmentfor hospitalized COVID-19 patients: A randomized, controlled clinical trial.Biomed. Pharmacother.2021, 111526

Souza, C.N. Almeida, A.C., Xavier, M.T.R., Costa, J.P.R., Silva, L.M.V., & Martins E.R. (2017). Antimicrobial activity of medicinal plants from the cerrado of Minas Gerais against bacteria isolated from sheep with mastitis. Unimonte scientific magazine. Montes Claros. 19 (2).

Stanaway, J.D., Shepard, D.S., Undurraga, E.A., Halasa, Y.A., Coffeng, L.E., Brady, O.J.,Hay, S.I., Bedi, N., Bensenor, I.M., Castaneda-orjuela, C.A., Chuang, T.W., Gibney, K.B., Memish, Z.A., Rafay, A., Ukwaja, K.N., Yonemoto, N., Murray, C.J.L., 2016. The global burden of dengue: an analysis from the Global Burden of Disease Study 2013. Lancet Infect. Dis. 16: 712–723.

Steyermark, J.A. 1974. Rubiaceae. In: T. Lasser (ed.). Flora de Venezuela: primeira parte 9: 1-593; secunda parte 9: 603-1101; tercera parte 9: 1111-2070.

Talipouo, A., Mavridis, K., Nchoutpouen, E. *et al.* High insecticide resistance mediated by different mechanisms in *Culex quinquefasciatus* populations from the city of Yaoundé, Cameroon. *Sci Rep* 11, 7322 (2021). https://doi.org/10.1038/s41598-021-86850-7

Tanya, S. ; Yordanov, D. Pseudomonas aeruginosa – a phenomenon of bacterial resistance. Med Microbiol. vol. 58 no. 9 1133-114. 2009.

Tinoco, CF, Lima NE, Lima-Ribeiro MS & Collevatti RG (2015) Research and partnerships in studies on population genetics of Neotropical plants: a scientometric evaluation. Biochemical Systematics and Ecology 61: 357-365

Tundisi JG & Tundisi TM (2008) Biodiversity in the Neotropics: ecological, economic and social values. Brazilian Journal of Biology 68: 913-915.

Turchetto-Zolet AC, Pinheiro F, Salgueiro F & PalmaSilva C (2013). Phylogeographical patterns shed light on evolutionary process in South America. Molecular Ecology 22: 1193-1213.

Valdes-Pena, M.A.; Massaro, N.P.; Lin, Y; Pierce, J.G. (2021). Leveraging Marine Natural Products as a Platform to Tackle Bacterial Resistance and Persistence. Accounts of Chemical Research. *54* (8), 1866-1877. DOI: 10.1021/acs.accounts.1c00007

Valentini, C.M.A.; Rodriguez-ortiz, C.E.; Coelho, M.F.B. 2010. *Siparuna guianensis* Aublet (negramina): uma Revisão. Rev. Bras. Plantas Med., v. 12, p. 96–104.

Vashist, S.K. In Vitro Diagnostic Assays for COVID-19: Recent Advances and Emerging Trends. *Diagnostics* 2020, *10*, 202. https://doi.org/10.3390/diagnostics10040202

Walker, M.J. ; Barnett, T.C.; McArthur, J.D. ; Cole, J.N. Disease manifestations and pathogenic mechanisms of Group A Streptococcus. Clin. Microbiol. Rev., 27 (2014), pp. 264-301

Weaver, S.C., Lecuit, M. 2015. Chikungunya virus and the global spread of a mosquito borne disease. New Engl. J. Med. 372 (13): 1231–1239.

Yang , X. , Yu , Y. , Xu , J. , Shu , H. , Liu , H. , Wu , Y. , Wang , Y. et al. (2020). Curso clínico e resultados de pacientes criticamente enfermos com pneumonia por SARS-CoV-2 em Wuhan, China: um estudo observacional, retrospectivo e centralizado . The Lancet Respiratory Medicine doi: https://doi.org/10.1016/S2213-2600(20)30079-5

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. New England Journal of Medicine, 382(8), 727–733. https://doi.org/10.1056/NEJMoa2001017

CHAPTER I - Antibacterial activity of *Siparuna guianensis* essential oil mediated by impairment of membrane permeability and replication of pathogenic bacteria

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ABSTRACT

The increasing prevalence of resistance to conventional antibiotics in pathogenic bacteria has demanded faster development of novel sources of antibacterial agents. In this context, biological activities shown by natural compounds (e.g., plant-based essential oils) have received particular attention. One of the alleged backslashes for these alternative bactericidal tools is the current knowledge gap regarding their action mechanisms. Here, we evaluated the activity of essential oil extracted of Negramina, *Siparuna guianensis*, plants against pathogenic bacteria (i.e., *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*). We evaluated cytotoxic effects on bacterial cells and the action mechanisms of the essential oil (and its major components). The gas chromatography analysis revealed that β-Myrcene (39.68%), epicurzerenone (18.16%) and germacrenes D (14.34%) and B (2.93%) are the major components of the *S. guianensis* essential oil. Interestingly, the essential oil showed negative effects on the four pathogenic bacteria without causing any toxicity to human monocytic cell line TPH-1 was exhibited. This antibacterial activity resulted from strong bacterial growth inhibition and deregulation of bacterial cell wall permeability with increased nucleotides and K^+ ions leakage. Furthermore, our molecular docking predictions indicated high affinity between some essential oil major components (e.g., Germacrene B) and active sites of bacterial DNA and RNA polymerases, which indicates possible impairments on the pathogenic bacteria cell replication processes.

Keywords: Bactericidal activity; Molecular docking; Germacrene B.

1 INTRODUCTION

Pathogenic bacteria like *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes* are ubiquitous microorganisms that survive under a variety of environmental conditions. Their importance derives from the health problems that they pose being agents of numerous diseases in humans and other warm-blooded animals (Oliveira et. al, 2019; Rahman et. al, 2018). Traditionally, the control of pathogenic bacteria is achieved using antimicrobial agents including antibiotic drugs and disinfectant agents with bactericidal effects.

Bactericidal agents induce bacterial cell death targeting a diverse set of biomolecules responsible for essential cellular processes. Exposure to bactericidal antibiotics has been shown to kill bacteria by inhibiting these cellular processes and by activating cellular

response pathways that contribute to cell death (Kohanski et al., 2010). Some of these processes, include DNA and RNA synthesis (DNA and RNA polymerases), protein synthesis (ribosomes), cell wall homeostasis (transglycosylases and peptidoglycan building blocks), as well as modulation of DNA topology (DNA topoisomerases) and penicillin-binding proteins (Kohanski et al.,2010; Daly et al.,2000; Sanyal et al., 2012).

On the other hand, bacterial resistance to antibiotics is becoming a global health issue (Scarafile, 2016) and the emergence of resistant bacteria is causing problems for both the treatment of patients and infections' control. In fact, some pathogenic bacteria have become resistant to entire classes of antibiotics, as in the case of *E. coli* resistance to ciprofloxacin (Wang et al., 2019), *S. aureus* resistance to methicillin (Krishnamoorthy et al, 2018), *P. aeruginosa* resistance to ampicillin (El-Banna et al, 2019) and *S. pyogenes* resistance to tetracycline (Kalumbi, 2019). Because of this rise and widespread of bacteria resistant to several drugs, bacterial infections have become major health challenges, generating increased interest in the search for and development of new antimicrobial agents.

Since antiquity, plants and their derivatives, including essential oils (EOs), have been used in folk medicine. Besides their important roles in the protection of plants, EOs present a broad range of secondary metabolites that are frequently reported to inhibit or slow the growth of bacteria, yeasts and molds (Nazzaro et al., 2013). Many studies have described the antimicrobial effects of EOs against both gram-positive bacteria such as *S. aureus* and *S. pyogenes* and gram-negative bacteria such as *E. coli* and *P. aeruginosa* (Lee et al, 2014; Tao et al, 2019).

The activity of EOs is known to depend on their chemical composition. The antimicrobial activity of EOs has a variety of targets in the bacterial cell membrane, and cytoplasm. They can disturb the cell permeability, damage the cell wall and the membrane proteins leading to leakage of the cell contents and alterations of the intracellular and external ATP balance (Nazzaro et al., 2013). EOs can also induce complete morphology change of the bacterial cells through alterations in its structure and functionality (Nazzaro et al., 2013).

Siparuna guianensis, popularly known as *Negramina,* is a Neotropical plant belonging to the Siparunaceae family that is recently drawing increasing interest for the aromatic, medicinal and biological properties of its essential oils and extracts. Products derived from its leaves, bark, and flowers have been widely used in folk medicine to treat sinusitis, fever, rheumatism, migraine, influenza, and body aches (Renner and Hausner, 2005; Valentini et. al, 2010). Recently, the essential oil of this plant species was also explored for its potential in insect pest management with promising results (Ferreira et al.,2017b; 2019; Lourenço et al., 2018). Moreover, compounds found in its essential oil including β-myrcene (Basera et al., 2019), germacrene D (Elshafie et al., 2019), germacrene B (Runyoro et al., 2010) and 2-undecanone are reported to have substantial antimicrobial potentials (Boudjema et al., 2018).

In the present study, we firstly investigated the antibacterial activity of the EOs of

S. guianensis against four species of pathogenic bacteria (i.e., *E. coli*, *S. aureus*, *P. aeruginosa* and *S. pyogenes*). Then, we assessed the cytotoxicity of *S. guianensis* essential oil both to bacterial cells and to human TPH-1 cells. Finally, we investigated the mechanisms behind the antibacterial activity at cells wall permeability level and we analyzed *in silico* molecular docking interactions of *S. guianensis* EO major compounds with bacterial DNA and RNA polymerases.

2 MATERIALS AND METHODS

2.1 Bacterial species

Antibacterial activity of *S. guianensis* EO compounds were evaluated against four bacterial species from the American Type Culture Collection (ATCC, Rockville, MD, US): *S. aureus* (ATCC 25923), *S. pyogenes* (ATCC 19615), *E. coli* (ATCC 35218) and *P. aeruginosa* (ATCC 2753). All strains were maintained on slopes of Skim Milk (Oxoids), stored at 20 °C, and subcultured two days before the assays to prevent morphological and metabolic transformations. Each strain was inoculated into 10 mL Mueller-Hinton broth and incubated at 37 °C under agitation of 200 rpm (Shaker Marconi – Mod. MA420, Brazil). After a 24-h incubation period, the suspensions were dispersed in sterile saline in glass tubes and stirred in a vortex until obtaining turbidity equivalent to the tube number 0.5 of the nephelometric McFarland scale (Lennet et al., 1985).

2.2 Plant material, essential oil extraction and gas chromatography–mass spectrometry (GC–MS) analysis

The leaves of *Siparuna guianensis* were collected and extraction of its essential oil was performed according to the methods described by Ferreira et al. (2017b) and, taxonomic identification was made by herbarium specialists from the Federal University of Tocantins (Porto Nacional-TO, Brazil - 10,496). The present investigation was registered in Brazil – SISGEN under number A7CAD12. The essential oils were extracted from *S. guianensis* leaves using the steam distillation method as described by (Aguiar et al., 2015, Lourenço et al., 2018). The gas chromatography–mass spectrometry (GC–MS) analysis was performed according to the methods described in Ferreira et al. (2017b), being the analysis of *S. guianensis* EO performed on the Chemito 8510 GC instrument (Chemito Technologies Pvt. Ltd, Mumbai, India) at the analytical center IQ-USP (São Paulo-SP, Brazil).

2.3 Antibacterial assays

2.3.1 Minimum inhibitory concentration (MIC)

To determine the minimum inhibitory concentration (MIC) of *S. guianensis* EO to *E. coli*, *S. pyogenes*, *P. aeruginosa* and *S. aureus*, the agar diffusion principle was used, according to the protocol recommended by CLSI (2005) with some adaptations. With suitably

sterilized swabs, the bacteria were homogeneously inoculated at concentrations of approximately 1.5 x 10^8 CFU / mL (Mac Farland 0.5 scale) in petri dishes containing Mueller Hinton Agar (MHA). Following the inoculation, concentrations of 0.87, 1.30, 1.70, 2.12, 17, 34, 68, and 102 µg/mL of the essential oil of *S. guianensis* were placed in 6-mm diameter wells as described by Ostrosky et al. (2008). After the 24-hour incubation period, the inhibition halos were measured in millimeters using a Tesa caliper. The lowest concentration of EO that inhibited the growth of the bacteria analyzed was considered the MIC.

2.3.2 Bacterial growth curve

The growth curves of *E. coli*, *S. pyogenes*, *P. aeruginosa* and *S. aureus* were evaluated following the method of Peyret et al. (1990). Briefly, bacterial samples were inoculated into Mueller Hinton broth and cultured for eight hours under stirring at 200 rpm and 37 °C. Then, increasing concentrations of *S. guianensis* EO (0.87, 1.30, 1.70, and 2.12 µg/ml) were homogenized using 200 µL of dimethylsulfoxide (DMSO) in 1 mL of the standardized (Mac Farland 0.5 scale) inoculum of bacterial samples and added to 20 ml of Mueller Hinton broth. The preparations were shaken (200 rpm) at 37 °C until measurements of optical densities. The control consisted of only 1 mL of standardized bacteria in 20 ml of Mueller Hinton broth and 200 µL of DMSO. The optical density readings at 600 nm were performed using a spectrophotometer (Quimis) at five intervals corresponding to 120, 210, 330, 390, and 430 minutes, after inoculation.

2.3.3 Intracellular K^+ efflux

The potassium efflux effect was measured according to the method of Arunachalam et al. (2016) with adaptations. Initially, *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* cells were cultured overnight at 37 °C. The cells were washed and resuspended at a concentration of 1 x 10^7 cells/mL in phosphate buffered saline (PBS) of pH 7.2. Subsequently, 1 mL of the bacterial suspensions of *S. guianensis* EO and rifamycin SV sodium (RSS) was incubated at 37 °C for different times. Bacterial strains incubated with PBS alone were used as controls. After centrifugation, the amounts of released K^+ in the supernatants were measured using a Microprocessor Flame Photometer (Quimis, Model Q498M2, São Paulo, SP, Brazil).

2.3.4 Nucleotide leakage

The nucleotide leakage was performed according to Arunachalam et al. (2016) with adaptations. Initially, *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* cells in logarithmic growth phase were washed and resuspended in 10 mM PBS (pH 7.2). The bacterial strains were incubated with *S. guianensis* EO and RSS for different times. Bacterial strains incubated with PBS alone served as the controls. The mixture absorbance was determined using a spectrophotometer at 260 nm (Shimadzu UV-1800, Vernon Hills, IL, US).

2.4 Cytotoxicity of *S. guianensis* EO to human TPH-1 cells

Essential oil of *S. guianensis* was dissolved in DMSO and diluted in RPMI culture medium (Sigma-Aldrich™) to form a stock solution. The cell viability test was performed using TPH-1 cells (ATCC® TIB-202™). Cytotoxicity was measured using the microplate dilution method. Once attached, the culture medium was removed and sample solutions were added at concentrations of 0.87, 1.30, 1.70, and 2.12 µg/mL. The final volume in each well was 100 µl and the quantity of cells present in each well was 1×10^4 cells. The plates were incubated for 48 h at 37 ºC in a humidified atmosphere with 5% CO_2. Next, 100 µl of MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) was added and incubated for 4 hours. Readings of absorbance at 540 nm were performed using a microplate spectrophotometer (Quimis). The assays were performed in triplicate and the results of the absorbances for each concentration were calculated according to the growth control. The CC_{50} (cytotoxic concentration at which 50% of the cells are viable) was calculated using dose-response graph nonlinear regression (Pillay et al., 2007). The cytotoxic assays were tested using ANOVA with a significance level of 5% by the Tukey method using OriginPro 8 software (OriginLab, 2007).

To visualize the potential cytopathic effects of *S. guianensis* EO on human monocytic cells, THP-1 cells were incubated with respective concentrations of *S. guianensis* in RPMI medium. Negative controls without the addition of the essential oil were used. After 48 hours, 10 µL of Hoechst 33342 were added and incubated at 37 °C for 15 minutes. The supernatants were discarded, and the wells were washed with PBS, followed by addition of 5 µL of propidium iodide (PI) for 20 minutes. Subsequently, the wells were washed with PBS and the circular coverslips containing the labeled cells were fixed with 3% formalin and cell viability was analyzed using a fluorescence microscope. Viable cells were indicated by blue fluorescence and non-viable cells were indicated by red fluorescence.

2.5 *In silico* studies of the interaction between the *S. guianensis* EO molecules and the receptor bacterial DNA and RNA polymerases

Amino acid sequences of DNA and RNA polymerases from *E. coli*, *S. pyogenes*, *P. aeruginosa* and *S. aureus* were obtained from the National Center for Biotechnology Information (NCBI) database. 3D structures of both proteins were constructed by homology modeling approach with The Swiss Model Workspace (https://swissmodel.expasy.org/), after the selection of its respective templates using BLASTp tool. The templates were downloaded from The Protein Databank (https://www.rcsb.org/), considering quality parameters as experimental method, resolution and R-value, as well as its complexing with a ligand. To check protein structure crashes and amino acid positioning in the active site, we used the Swiss model (Waterhouse, 2018). Validation of the generated models was performed by inspection of the Ramachandran plots (Ramachandran, 1968; Haas et. al, 2017), in which it was possible

to analyze the distribution of the torsion angles of the backbone ϕ and ψ responsible for the stereochemical quality of the protein studied as well as the QMEAN factor (Benkert et al, 2011).

Nine docking positions were generated for each ligand interacting with bacterial DNA and RNA polymerases, returning affinity energy values (Kcal / Mol) using the AutoDock Vina (Trott and Olson, 2010) in the docking calculations, initially the *Siparuna guianensis* molecules designed with Marvin Sketch 18.10 (ChemAxon) and receptors and ligands prepared for the molecular docking process using Autodock Tools 1.5.7 (Sanner, 1999), according to the methodology proposed by Borges et al. (2019).

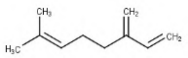
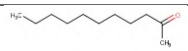
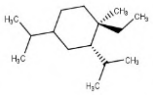
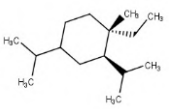
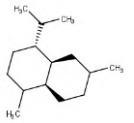
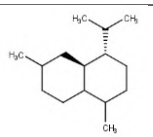
The docking position results were analyzed using PyMOL 2.0 (Schrodinger, 2018) and Discovery Studio 4.5 (Dassault Systemes BIOVIA, 2017) for selecting the best position for each ligand inside the protein target using the parameters proposed by Borges et al. (2019).

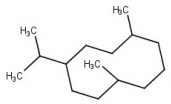
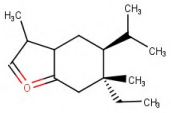
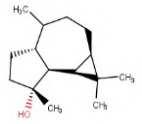
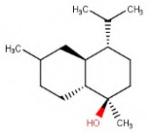
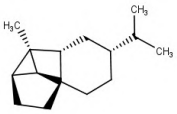
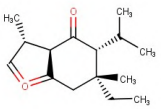
3 RESULTS

3.1 Chemical composition of the EO from *S. guianensis*

The composition and identification of the main compounds present in EO of *S. guianensis* in GC-MS analyses revealed 12 compounds (Table 1). The major components from the leaves were β-myrcene with 39.67%, germacrene D with 14.34% and epicurzerenone with 18.16%. We also detected germacrene B at 2.93%**.**

Table 1. Chemical composition of *Siparuna guianensis* essential oil

Compound	Molar mass	Molecular formula	Chemical structure	%	Ric*
β-Myrcene	136.2	$C_{10}H_{16}$		39.67	986
2-Undecanone	170.2	$C_{11}H_{22}O$		6.25	1251
γ-Elemene	204.3	$C_{15}H_{24}$		3.5	1439
Elixene	204.3	$C_{15}H_{24}$		3.5	1435
γ-Muurolene	204.3	$C_{15}H_{24}$		1.34	1441
δ-Cadinene	204.3	$C_{15}H_{24}$		1.25	1478

Compound	Molar mass	Molecular formula	Chemical structure	%	Ric*
Germacrene D	204.3	$C_{15}H_{24}$		14.34	1528
Curzerene	216.3	$C_{15}H_{20}O$		4.91	1542
Spathulenol	220.3	$C_{15}H_{24}O$		1.03	1547
α-Cadinol	222.3	$C_{15}H_{26}O$		1.32	1592
Germacrene B	204.3	$C_{15}H_{24}$		2.93	1613
Epicurzerenone	230.3	$C_{15}H_{24}O_2$		18.16	1611
Not identified (%)	-	-	-	1.80	-

* Ric = Retention index calculated

3.2 Determination of minimum inhibitory concentration (MIC)

The MIC results among the tested concentrations of *S. guianensis* EO are presented in Table 2. The low concentrations for the MIC suggest that the bacterial species used are highly susceptible to the effect of *S. guianensis* EO, with MIC values ranging from 0.86 µg/mL (*E. coli*, *S. pyogenes*, *P. aeruginosa*) to 1.30 (*S. aureus)*.

Table 2. Antimicrobial activity of the *S. guianensis* EO expressed as minimum inhibitory concentration (MIC).

Bacteria	GRAM	MIC value (µg/mL)
S. aureus	+	1.30 a
E. coli	-	0.87 b
P. aeruginosa	-	0.87 b
S. pyogenes	+	0.87b

3.3 Growth curve of the bacteria against *S. guianensis* EO

Optical absorbances of bacterial cultures over time in the presence of increasing concentrations of *S. guianensis* EO (Figure 1) showed that even the lowest concentration (0.87 µ /mL) was extremely toxic and inhibited cell growth of *E. coli, P. aeruginosa* and *S. pyogenes.* The same concentration slowed the cell growth of *S. aureus* with OD reaching only 0.332 after 430 minutes. Higher concentrations (1.70 and 2.12 µg/ml) completely inhibited the growth.

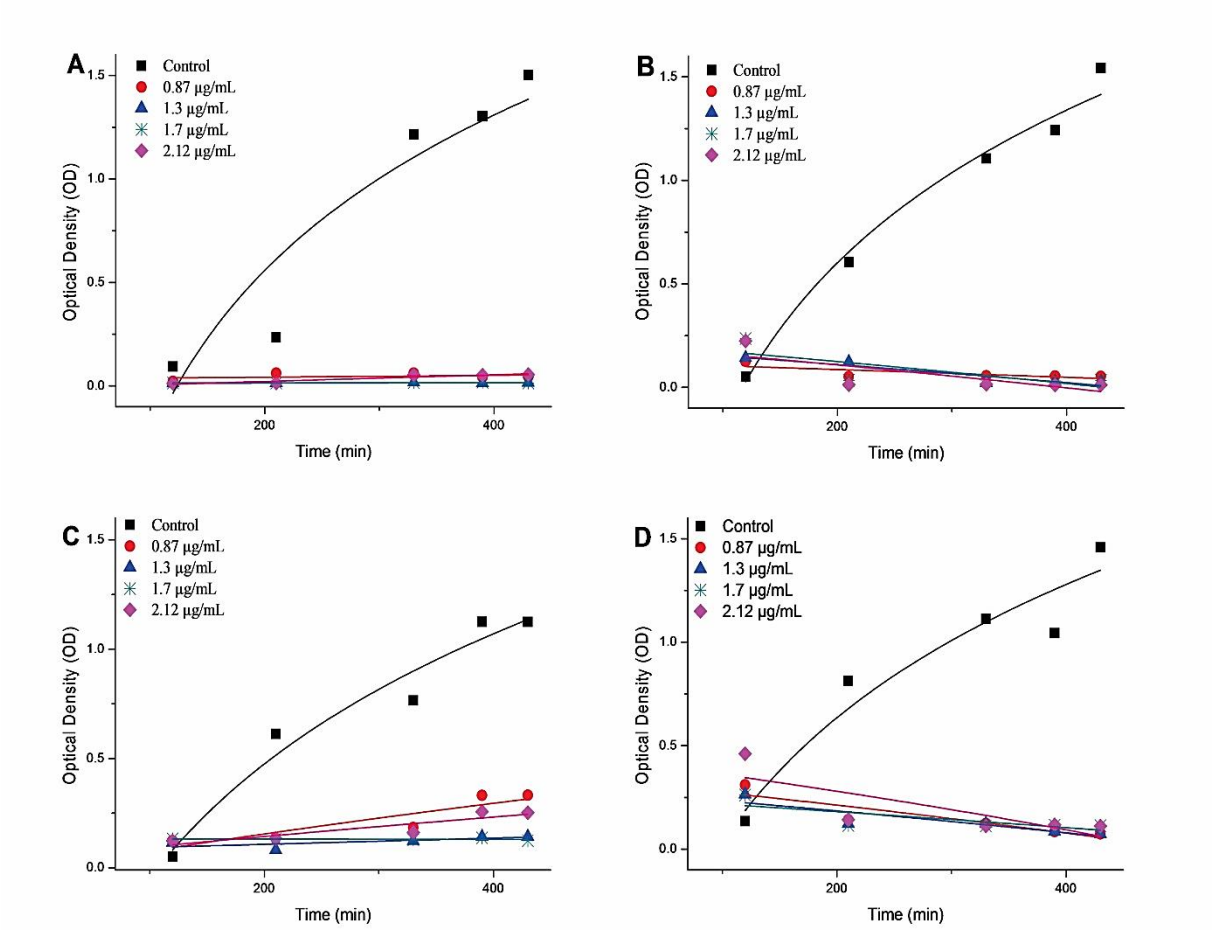


Figure 1. Effect of EO *S. guianensis* on the determination of growth curves (A) *E. coli*, (B) *P. aeruginosa*, (C) *S. aureus* and (D) *S. pyogenes.*

3.4 K^+ efflux induced from *S. guianensis* EO

Potassium efflux independently increased during the contact period of the S. guianensis EO with bacteria strains. We observed that the maximum value was found at the 2nd hour for *S. aureus* (4.3 ppm), at the 3rd hour for *S. pyogenes* (4.8 ppm), and 4th hour for *E. coli* and *P. aeruginosa* (4.4 and 5.4 ppm, respectively), while the control showed a slight variation of only 0.3 ppm throughout the experiment. For the RSS antibiotic, we found that *S. aureus* had its highest value at the 3rd hour (4.4 ppm) and *E. coli*, *S. aureus* and *S. pyogenes* had higher values for potassium efflux at the 4th hour (3.7, 4.2 and 4.2 ppm, respectively) (Figure 2).

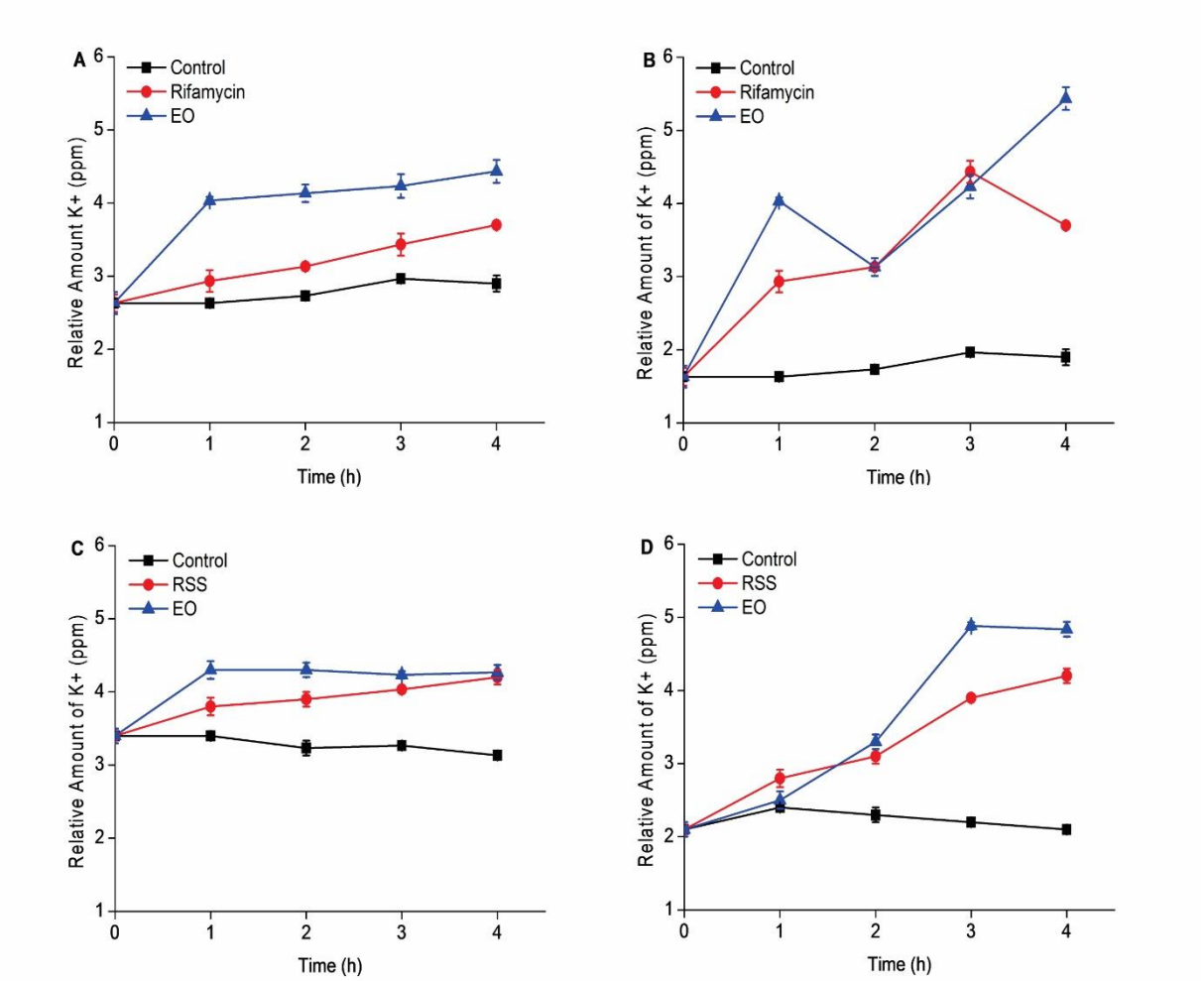


Figure 2. Effect of MIC of EO *S. guianensis* on the amount K^+ release from (A) *E. coli*, (B) *P. aeruginosa*, (C) *S. aureus* and (D) *S. pyogenes*. Each value represents mean ±SD; three independent experiments.

3.5 Nucleotide leakage

There was considerable nucleotide leakage in all bacteria tested, starting from the 2nd hour with the greatest effect for the test in the presence of *S. guianensis* EO with *E. coli* (0.477), *P. aeruginosa* (0.575), *S. aureus* (0.568) and *S. pyogenes* (0.522), when compared to the RSS antibiotic that showed values of 0.376, 0.392, 0.157 and 0.257, respectively (Figure 3). There was very little variation of the control throughout the experiment.

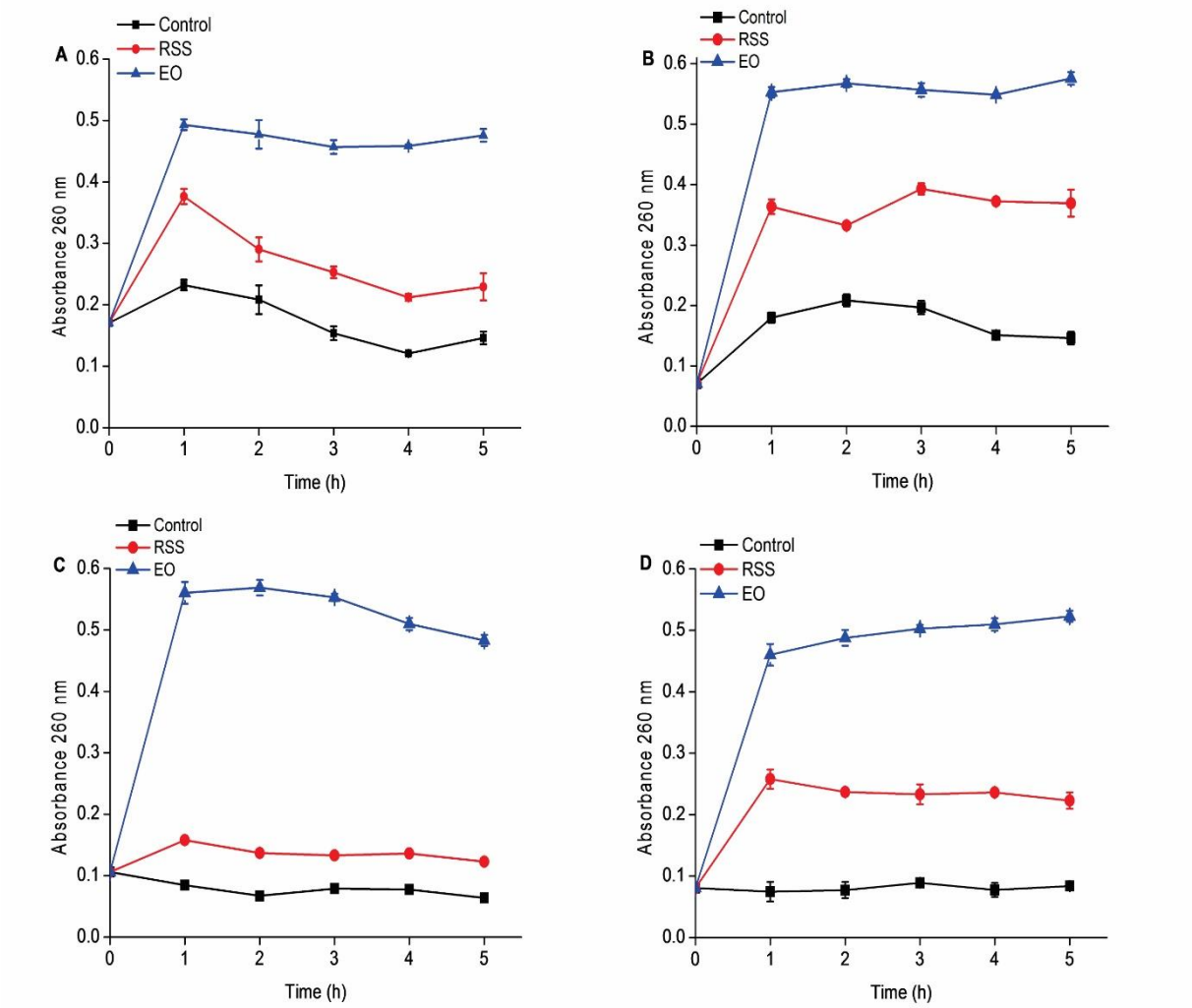


Figure 3. Effect of MIC of EO *S. guianensis* on the amount total nucleotide release from (A) *E. coli*, (B) *P. aeruginosa*, (C) *S. aureus* and (D) *S. pyogenes*. Each value represents mean ±SD; three independent experiments.

3.6 Cytotoxicity of *S. guianensis* EO to TPH-1 cells (human monocytic cells)

We tested toxicity of *S. guianensis* EO in human monocytic cells (TPH1 cell line) at 0.87, 1.30, 1.70, and 2.12 µg/ml. None of these concentrations caused significantly more toxicity than controls (Figure 4). The concentrations of *S. guianensis* EO that showed inhibitory effects on *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* bacterium were not toxic for TPH1 cells.

Similar to the results of the MTT cytotoxicity test, cell viability analyses using immunofluorescence indicated that no concentration of *S. guianensis* EO generated significant toxic effects (Figure 4). In all analyzed fields, there was a predominance of THP-1 cells with blue fluorescence.

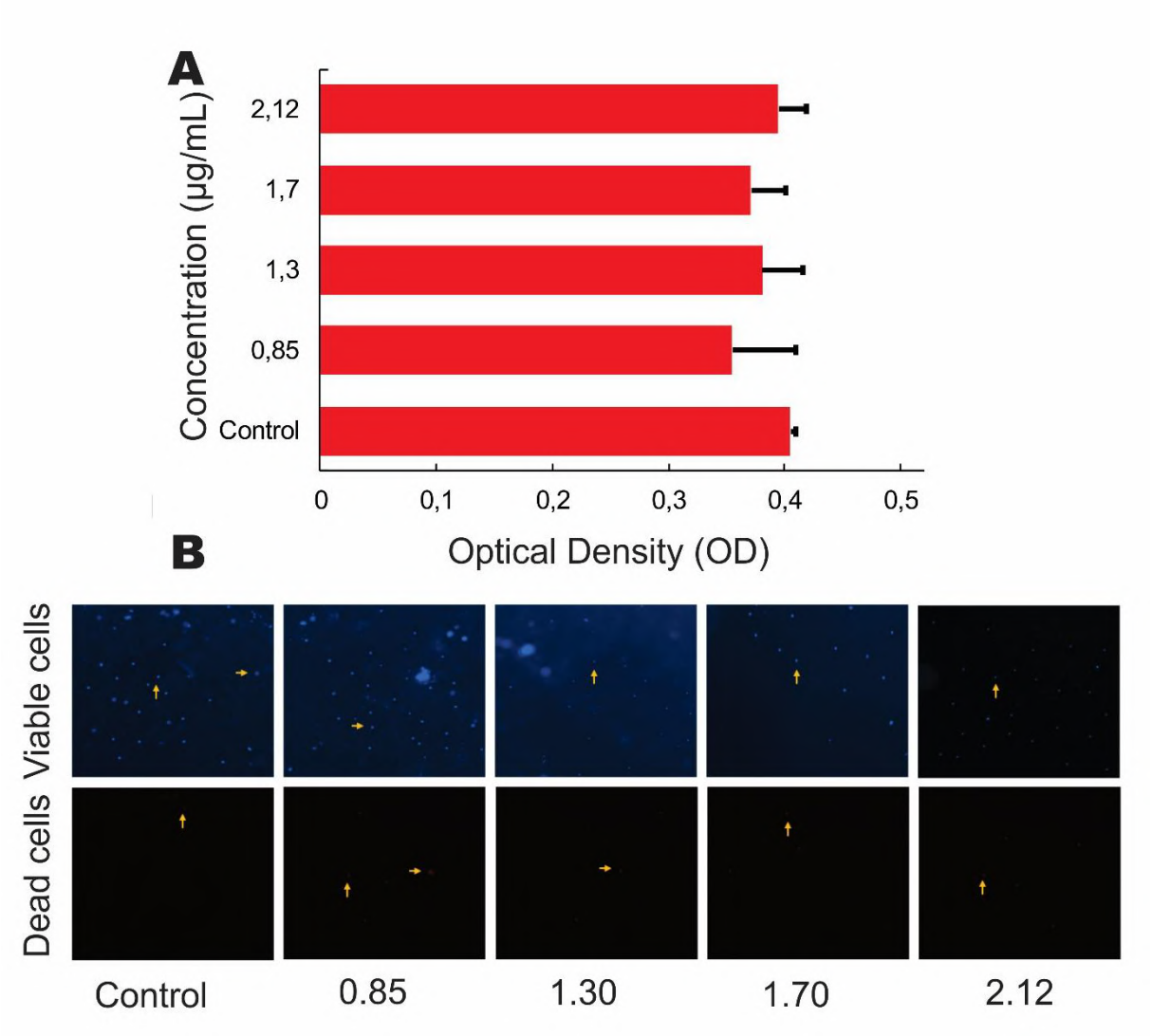


Figure 4. Cytotoxicity test with human monocytic TPH-1 cells submitted to EO *S. guianensis* at concentrations of 0.87, 1.30, 1.70 and 2.12 µg/mL analyzed by a) absorbances and b) immunofluorescence (Cells analyzed in 10x size with filters to detect blue (viable) and red fluorescence (dead))

3.7 Interaction of *S. guianensis* EO molecules and bacterial DNA and RNA polymerases

According to the results obtained from bacterial bioassays, our hypothesis is that main compounds present in *S. guianensis* EO could interact with the enzymes' DNA and RNA polymerases of these bacteria, inhibiting replication. Table 3 shows the selected templates for homology modeling, highlighting the identities and the validation results with the corresponding Ramachandran favored values.

The compounds present in *S. guianensis* EO complexed with the different receptors and formed various types of interactions with varying affinity energies as indicated by the docking assays performed (Table 4). Besides all complexed ligands, germacrene B presented better affinity energy with *E. coli*, *P. aeruginosa* and *S. aureus* DNA and RNA

polymerases (Figures 5A, 5B, 5C, 6A, 6B and 6C, respectively), as well as complexed with the RNA polymerase of *S. pyogenes* (Figure 6D).

Germacrene B complex with E. coli DNA polymerase showed interactions with active site amino acids and the ligand: alkyl-type interactions with PHE228, MET314 and PRO710 and van der Waals interactions with ASP156 ILE157, GLU158, THR159, TRP223, ASN224, LYS305 and ARG313 (Figure 5D). For the RNA polymerase, we found alkyl interactions with PHE14 and MET11, and van der Waals interactions with ASN10 and GLN18 (Figure 6E). The complex formed between germacrene B and *P. aeruginosa* DNA polymerase presented alkyl-type interactions with VAL427, TYR430 and PHE436, and van der Waals interactions with TYR590 (Figure 5E). Additionally, germacrene B complexed with *P. aeruginosa* showing alkyl interactions with ALA157, PHE169, PHE171, VAL177, ALA189, PHE207 and ILE331, and van der Waals interactions with PHE140, GLU170 and LEU193 (Figure 6F).

DNA polymerase of *S. aureus* receptors complexed with germacrene B showing alkyl-type interactions with at LEU583, LEU584, TYR588, PRO627, VAL628 and ARG629, as well as Van deer Walls with GLN580, ASN625, ILE626, LEU630, GLU632 and GLY633 (Figure 5F). On the other hand, the complex with RNA polymerase presented only one alkyl interaction with PHE14, and van der Waals interactions with ASN10, MET11 and GLN18 (Figure 6G).

Due to low identity between target and templates, it was not possible to construct the DNA polymerase structure from *S. pyogenes*. However, we constructed a reliable 3D structure of RNA polymerase, and its interaction with germacrene B showed alky interactions with TYR62, TYR65, PRO70 and VAL172, and van der Waals interactions with VAL173 (Figure 6H).

For all targets, we performed a re-docking with rifamycin SV sodium as a positive control. For all docking results, germacrene B occupied the same region of the ligand control (Supplementary Figure 1).

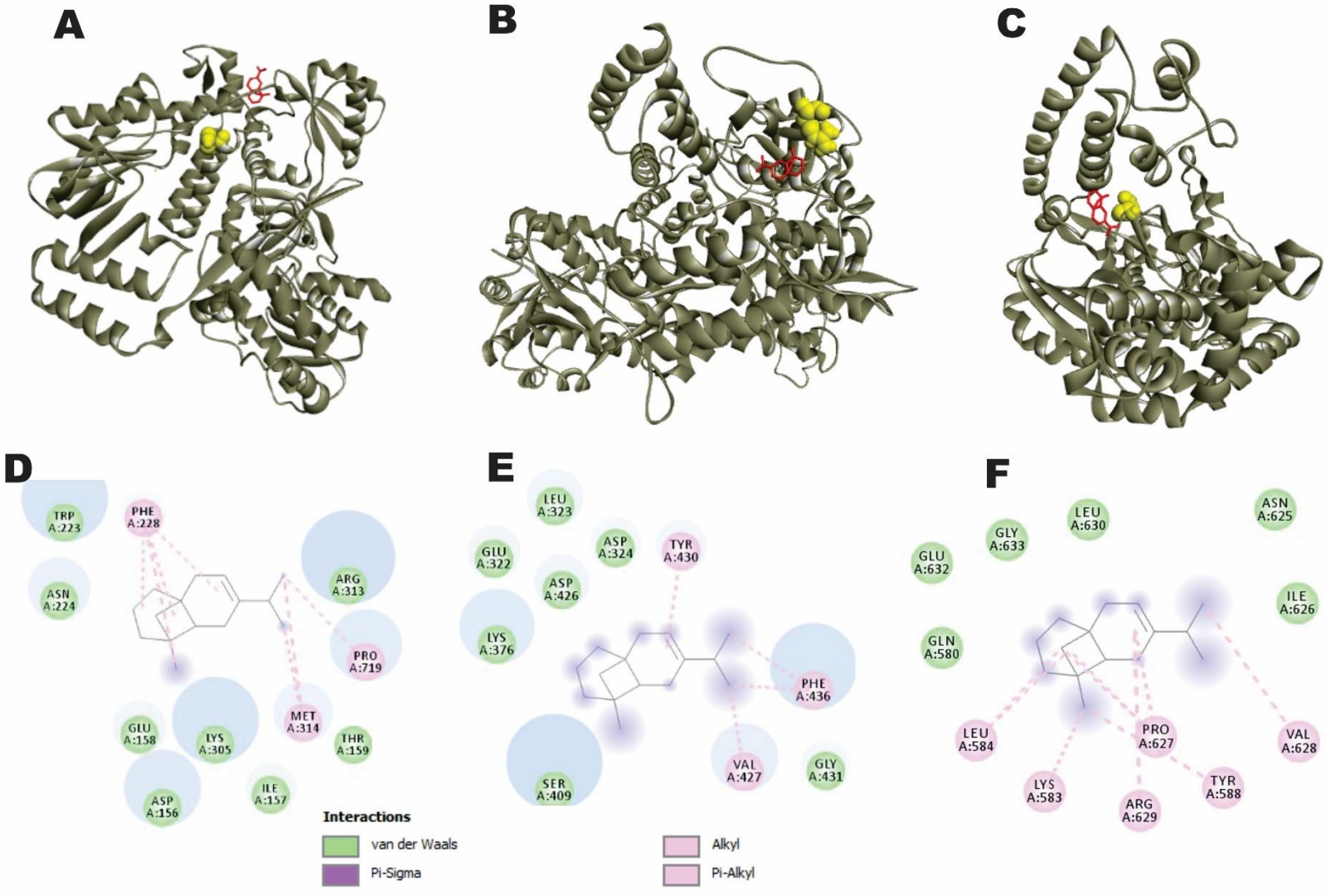


Figure 5. Germacrene B (Red) complexed with DNA Polymerase enzyme (A, B, C) and 2D maps of molecular interactions with amino acids in DNA Polymerase Active site (Yellow) (D, E, F) of *E. coli* (A, D)*, P. aeruginosa* (B, E), *S. aureus* (C, F) and of *E. coli* (D)*, P. aeruginosa* (E) and *S. aureus* (F).

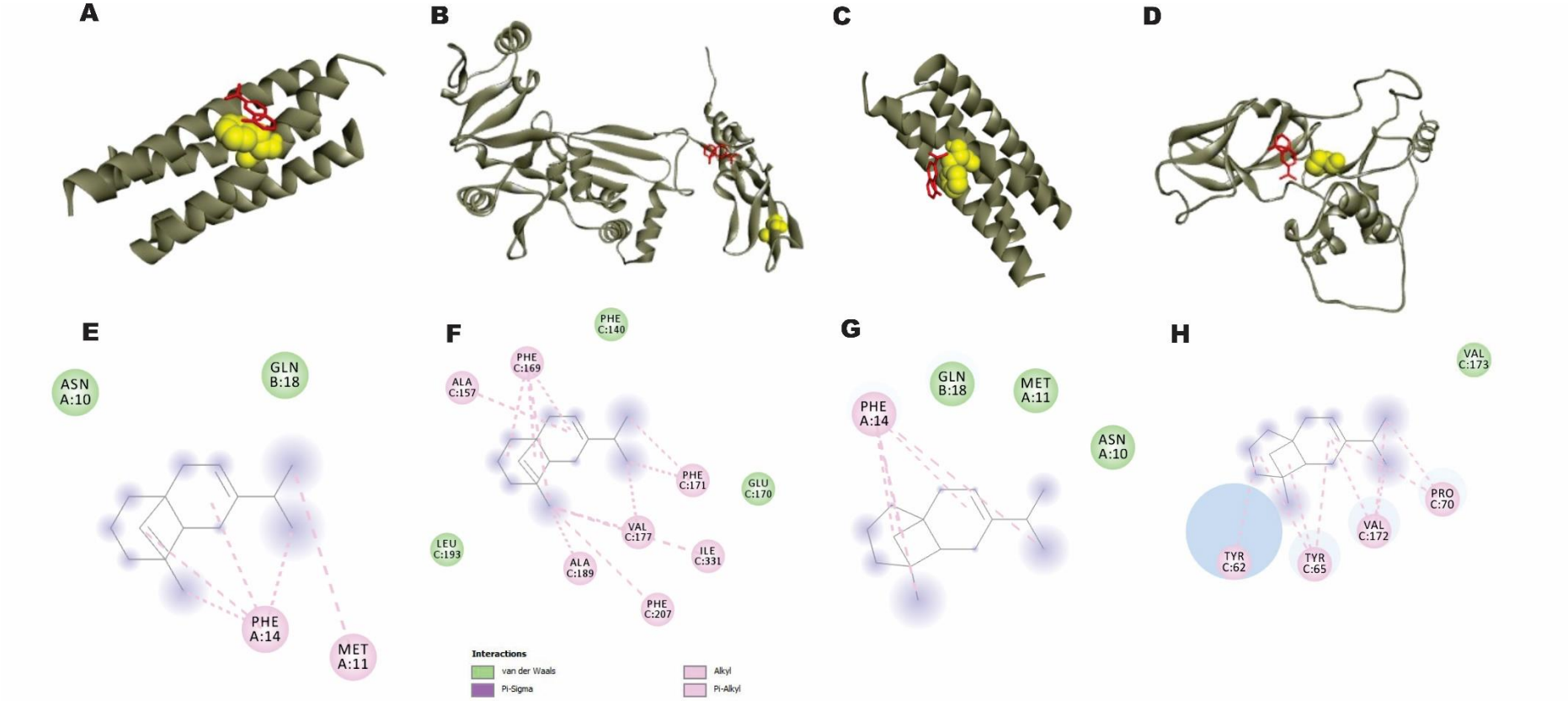


Figure 6. Germacrene B (Red) complexed with RNA Polymerase enzyme (A, B, C, D) and 2D maps of molecular interactions with amino acids in RNA Polymerase Active site (Yellow) (E, F, G, H) of *E. coli* (A, E)*, P. aeruginosa* (B, F), *S. aureus* (C, G) and *S.pyogenes* (D, H).

4 DISCUSSION

The *Siparuna guianensis* EO had β-myrcene as its primary component, this is a bactericidal molecule for human pathogens such as *Streptococcus pneumonia*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* (Skocibusic, 2004). The EO also contains germacrenes D and B, found also in the EO of *Copaifera officinalis* that has bactericidal activity for *S. aureus* and *E. coli* (Andrade et al, 2013). Other authors have found various chemical elements in varying proportions than those found in the present study (Andrade et al., 2013, Ferreira et al., 2017a), suggesting that the composition of *S. guianensis* EO depends on its location, as well as on the period in which the plant was collected and its oil was extracted.

Antibacterial activity of compounds is considered significant when the MIC is below 10 µg/mL (Omosa et al., 2016). This suggests that MIC values ranging from 0.86 to 1.30 µg/ml for *S. guianensis* EO against the bacteria used in this study were effective. In the EO of *Rosmarinus officinalis* obtained by hydrodistillation, the MIC value was 3.75 µg/mL for *S. aureus* and 7.5 µg/mL for *E. coli* (Probst, 2012). In assays using *A. herba-alba* performed by Sbayou et al. (2014), the MIC value was against *E. coli* was 1.25 µg/mL. Mighri et al. (2010) reported MIC of 0.62 mg/mL against *S. aureus*, a value higher than found in the present study, where the essential oil of *S. guianensis* showed strong antibacterial effects against both gram-positive and gram-negative bacteria.

We observed that while the control samples showed expected growth rates, the samples treated with the EO showed growth inhibition. The EO prevented bacterial replication at all concentrations tested. According to Lin et al. (2018), OD values are influenced by membrane permeability of the bacteria; this suggests that, after treatment with *S. guianensis* EO, we observed a reduction in OD values, characterizing greater permeability of EO into the cellular structure of the bacteria.

The decrease in OD values for the various bacteria in contact with *S. guianensis* EO at various time points of the experiment suggests bactericidal effects, as proposed by Nocchetti et al. (2013). In the same sense, Bachir et al. (2012) observed inhibition of growth of *S. aureus* and *E. coli* caused by *E. globulus* EO measured in terms of optical density at various concentrations of essential oil, and *S. aureus* growth was totally inhibited when 50 µL of the oil was used within 2 hours of exposure. At 100 µL of the oil, 10 min exposure inhibited the growth of the bacteria for all amounts of broth dilution.

Comparatively speaking, all the concentrations used in this work were low, compared to those used in other works, such as those of Xu et al. (2018), who observed inhibition halos at 12.7 µg/mL of *Artemisa Asian* EO against *S. aureus*, 14.3 µg/mL against *S. pyogenes*, and 9.2 µg/mL against *P. aeruginosa*, which demonstrates the good effect provided by the EO of *S. guianensis.*

Transport of K^+ ions is extremely important for bacterial pathogenesis via regulation of cytoplasmic and cellular pH, primarily mediated by efflux control, as explained by Roosild et

al. (2010). Unregulated leakage of ions such as potassium suggests damage that EO causes to the cytoplasmic membrane, allowing exposure of vital intracellular materials. We also observed that the nucleotide leakage analysis showed that *S. guianensis* EO increased cell wall permeability, causing extracellular nucleotide leakage, further facilitating the interaction of EO compounds with bacterial replication receptors, as shown, and as described by Omelon et al. (2016).

Studies have been proposed to determine the cytotoxicity of essential oil compounds in human cells, as well as their use as antibacterial agents, including the essential oil of *Minthostachys verticilata* (perperina) (Escobar et al., 2012). We found that the essential oil of *S. guianensis* did not induce cytotoxic effects, as indicated by the MTT assay in TPH1 cells, as well as by immunofluorescence, suggesting that *S. guianensis* essential oil could be safely used as a therapeutic agent.

Florão (2006) reported that the essential oils of *Baccaris dracunculifolia*, *B. cirspa*, *B. gaudichaudiana* and *B. articulata* presented high cytotoxicity at 10 µL/mL for mononuclear cells and granulocytes. In the present study, the concentrations used were not toxic to TPH-1 cells when compared to controls. The concentrations of essential oil that showed better inhibitory effects for *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes*, showed no toxicity in TPH-1 cells.

For a better understanding of the mechanism of interaction between *S. guianensis* EO and bacteria, possible receptors were selected based on the hypotheses formed in the present study, specifically that the bacteria treated with the EO did not replicate. Hu et. al (2019) found that *Litsea cubeba* EO influenced bacterial DNA and RNA replication in methicillin-resistant *Staphylococcus aureus*. Fang et al. (2016) showed through molecular docking that some EO flavonoids can interact with *E. coli* DNA.

The *S. guianensis* EO compounds that interact with the active site regions promoted by docking with bacterial DNA and RNA polymerases promote a change in conformation, thereby inhibiting replication. DNA replication, transcription and translation operate with impressive speed and fidelity in bacterial cells; however, these properties are influenced by the action of polymerases (Robinson and Van Oijen, 2013). A change in the conformation of these enzymes can cause great variations in reaction rates, and may even inhibit bacterial replication.

In all living organisms, genomes are replicated by DNA polymerase expressed from the genomes themselves via transcription and translation. This process may undergo changes as a function of external interferences mainly in bacteria (Fujiwara et al, 2013). The DNA and RNA polymerase structures of the bacteria were then modeled as receptors of *S. guianensis* EO ligands. Almost all of them presented identity superior to 30% as indicated by the literature (Xiang, 2006). It was not possible to model DNA polymerase of *S. pyogenes*, which did not have satisfactory identity for homology modeling. The models had

Ramachandran favored results above 90% (Giacoppo et al., 2016).

In the present study, we found that the selected molecular targets of *E. coli*, *S. aureus* and *P. aeruginosa* had better values for energy affinity for germacrene B, and only *S. pyogenes* presented better values for α-cadinol, with values very close to those of germacrene B, according to values described in the literature (Shityakov and Förster, 2014). Other authors also reported the bactericidal activity of several EOs containing germacrene B (Oyedeji et al., 2005, Runyoro et al., 2010, Andrade et al., 2013), suggesting that this molecule has bactericidal action, also considering its interaction in the same region of the antibiotic RSS (Ganapathy et al., 2019).

Essential oils have been widely used in various alternative treatments. They inhibit the growth of a wide range of pathogens, mediated by natural compounds produced by plant organs (Swamy et al., 2016). iI is important to note that the unique aroma and other bioactive properties of EOs depend on their chemical constituents.

5. CONCLUSION

There is increased interest in the use of EOs for control of pathogenic bacteria, such as *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes*. However, quantitative and qualitative variations in the chemical composition is still a complicating factor against the use of EOs that have bactericidal effects. Therefore, it is extremely important to identify the main compounds present in these Eos as well as to understand their mechanisms of action and interactions with vital processes of these organisms in order to better explore their bactericidal activity. S. guianensis EO showed good bactericidal activity, increasing the cell wall permeability of bacteria, and molecular docking analysis showed that germacrene B was a highly reactive molecule when compared to the reference antibiotic. Such knowledge will surely increase the list of products of botanical origin that can be used to reduce the impact of the diseases caused by such pathogenic microorganisms.

REFERENCES

Aguiar, R. W. S., Santos, S. F., Morgado, F. S., Ascencio, S. D., Lopes, M. M., Viana, K. F., Didonet, J., & Ribeiro, B. M., 2015. Insecticidal and repellent activity of *Siparuna guianensis* Aubl. (negramina) against *Aedes aegypti* and *Culex quinquefasciatus*. *Plos One* 10(2): e0116765. https://doi:10.1371/journal.pone.0116765.

Alhazmi, A., 2015. *Pseudomonas aeruginosa* – Pathogenesis and Pathogenic Mechanisms. I. J. of Biol. 7(2). https://doi:10.5539/ijb.v7n2p44

Andrade, B. F. M. T., Barbosa, L. N., Probst, I. S., Júnior A. F., 2013. Antimicrobial activity of essential oils. *J. Essent. Oil Res*. 26(1), 34–40.

Arunachalam, K., Ascêncio, S. D., Soares, I. M., Souza Aguiar, R. W., da Silva, L. I., de Oliveira, R. G., de Oliveira Martins, D. T., 2016. *Gallesia integrifolia* (Spreng.) Harms: In vitro

and in vivo antibacterial activities and mode of action. J. Ethnopharmacol. 184, 128–137. https://doi:10.1016/j.jep.2016.03.005

Bachir, R.G., Benali, M., 2012. Antibacterial activity of the essential oils from the leaves of *Eucalyptus globulus* against *Escherichia coli* and *Staphylococcus aureus*. Asian Pac. J. Trop. Biomed. 2(9), 739–742. https://doi:10.1016/S2221-1691(12)60220-2

Baracco, G. J., 2019. Infections caused by group C and G Streptococcus (*Streptococcus dysgalactiae* subsp. equisimilis and Others): Epidemiological and clinical aspects. Microbiol. Spectr. 7(2). https://doi:10.1128/microbiolspec.gpp3-0016-2018

Barbosa-JR, F., Fillion, M., Lemire, M., Passos, C.J., Rodrigues, J.L., Philibert, A., Guimarães, J.R., Mergler, D., 2009. Elevated blood lead levels in a riverside population in the Brazilian Amazon. Environ. Res. 109 (5), 594-599.

Basera, P., Lavania, M., Agnihotri A., Lal, B., 2019. Analytical investigation of *Cymbopogon citratus* and exploiting the potential of developed silver nanoparticle against the dominating species of pathogenic bacteria. Front Microbiol. 10, 282. https://doi:10.3389/fmicb.2019.00282

Benkert, P., Biasini, M., Schwede, T., 2011. Toward the estimation of the absolute quality of individual protein structure models. Bioinform. 27, 343-350.

Borges, J.C.M., Haddi, K., Oliveira, E.E., Andrade, B.S., Nascimento, V.L., Melo, T.S., Didonet, J., Carvalho, J.C.T., Cangussu, A.S., Soares, I.M., Ascencio, S.D., Raposo, N.R.D., Aguiar, R.W.S., 2019. Mosquiticidal and repellent potential of formulations containing wood residue extracts of a Neotropical plant, *Tabebuia heptaphylla*. Ind. Crops. 129, 424-433. https://doi.org/10.1016/j.indcrop.2018.12.022

Boudjema, K., Guerdouba, A., Hali, L., 2018. Composition, physicochemical analysis, antimicrobial and anti- inflammatory activities of the essential oils obtained from ruta chalepensis l growing wild in northen of Algeria. J. Chem. Soc. Pak. 40(6), 1054-1062.

Carmona-Torre, F., Torrellas, B., Rua, M., Yuste, J. R., & Del Pozo, J. L., 2017. *Staphylococcus aureus* nasal carriage among medical students. Lancet Infect. Dis. 17(5), 477–478. https://doi:10.1016/s1473-3099(17)30188-3

Chouhan, S., Sharma, K., Guleria, S., 2017. Antimicrobial activity of some essential oils-present status and future perspectives. Med. (Basel). 4(3), 58. https://doi:10.3390/medicines4030058

Clinical and laboratory standards institute/NCCLS. Performance Standards for Antimicrobial Susceptibility Testing; Fifteenth Informational Supplement. CLSI/NCCLS document M100-S15 [ISBN 1-56238-556-9]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2005.

Daly, J.S., Giehl, T.J., Brown, N.C., Zhi, C., Wright, G.E., Ellison, R.T., 2000. In vitro antimicrobial activities of novel anilinouracils which selectively inhibit DNA polymerase III of gram-positive bacteria. Antimicrob. agents and chemother. 44(8), 2217-2221.

Dassault Systemes BIOVIA, 2017. Discovery Studio Modeling Environment, Release 2017. Dassault Systemes, San Diego.

El-Banna, T., Abd El-Aziz, A., Sonbol, F., El-Ekhnawy, E., 2019. Adaptation of *Pseudomonas aeruginosa* clinical isolates to benzalkonium chloride retards its growth and enhances biofilm production. Mol. Biol. Rep. https://doi:10.1007/s11033-019-04806-7

Elshafie, H.S., Gruľová, D., Baranová, B., Caputo, L., De Martino, L., Sedlák, V., Camele, I., De Feo, V., 2019. Antimicrobial activity and chemical composition of essential oil extracted from *Solidago canadensis* L. growing wild in Slovakia. *Molecules. 24*, 206. https://doi.org/10.3390/molecules24071206

El-Shouny, W.A., Ali, S.S., Sun, J., Samy, S.M., Ali, A., 2018. Drug resistance profile and molecular characterization of extended spectrum beta-lactamase (ESβL)-producing *Pseudomonas aeruginosa* isolated from burn wound infections essential oils and their potential for utilization. Microb. Pathog. https://doi: 10.1016/j.micpath.2018.02.005.

Escobar, S., Espigares puerto, F., Felip, A., Zanuy, S., Gueguen, M.M., Carrillo, M., Kah, O., Servili, A., 2012. Kisspeptin/GPR54 expression in the brain of the European sea bass. Abstract book of the 7th International Symposium of Fisch Endocrinilogy. Buenos Aires, Argentina. 152.

Fang, Y., Lu, Y., Zang, X., Wu, T., Qi, X., Pan, S., & Xu, X., 2016. 3D-QSAR and docking studies of flavonoids as potent *Escherichia coli* inhibitors*.* Scientific. Rep. *6(1).* https://doi:10.1038/srep23634

Ferreira, T. P., Kuckelhaus, I. G. P., Tschoeke, P. H., Cangussu, A. S., Borges, J. C. M., Moura, W. S., Aguiar, R. W. S. 2017a. Influence of seasonality on the yield and composition of the essential oil of *Siparuna guianensis* Aublet. African J. Biotechn. 16(29), 1611–618. https://doi:10.5897/ajb2017.16109

Ferreira, T. P., Oliveira, E. E., Tschoeke, P. H., Pinheiro, R. G., Maia, A. M. S., & Aguiar, R. W., 2017b. Potential use of Negramina (*Siparuna guianensis* Aubl.) essential oil to control wax months and its selectivity in relation to honey bees. Ind. Crops Prod*.* 109, 151-157. https://doi.org/10.1016/j.indcrop.2017.08.023

Ferreira, T.P., Haddi K, F. T., Corrêa, R., Zapata, V.L.B., Piau, T.B., Souza L.F.N., Aguiar, R.W.S., 2019. Prolonged mosquitocidal activity of *Siparuna guianensis* essential oil encapsulated in chitosan nanoparticles. PLos Negl. Trop. Dis. 13(8), e0007624. https://doi.org/10.1371/journal. pntd.0007624

Florão, A., 2006. Avaliação de Atividades Biológicas de óleos essenciais de quatro espécies de *Baccharis arteraceae*. Dissertação de mestrado. Programa de Pós-Graduação em Ciencias Farmaceuticas- Universidade Federal do Paraná. Curitiba.

Fujiwara, K., Katayama, T. & Nomura, S. M., 2013. Cooperative working of bacterial chromosome replication proteins generated by a reconstituted protein expression system. Nucleic. Acids *Res.* 41, 7176-7183. https://doi.org/10.1093/nar/gkt489.

Ganapathy, U.S., Dartois, V., Dick, T. 2019. Repositioning rifamycins for Mycobacterium abscessus lung disease. Exp. Opin. Drug. Discov. 14 (9), 867-878. https://doi.org/10.1080/17460441.2019.1629414

Giacoppo, J. de O. S., Carregal, J. B., Junior, M. C., Cunha, E. F. F. da, & Ramalho, T. C., 2016. Towards the understanding of tetrahydroquinolines action in *Aedes aegypti:* larvicide or adulticide? Mol. Simul. 43(2), 121–133. https://doi:10.1080/08927022.2016.1239823

Gomes, T. A. T., Elias, W. P., Scaletsky, I. C. A., Guth, B. E. C., Rodrigues, J. F., Piazza, R. M. F., Martinez, M. B., 2016. Diarrheagenic *Escherichia coli*. Braz. J. Microb. 47, 3–30. https://doi:10.1016/j.bjm.2016.10.015

Gutiérrez, Y., Scull, R., Villa, A., Satyal, P., Cos, P., Monzote, L., & Setzer, W., 2019. Chemical composition, antimicrobial and antiparasitic screening of the essential oil from *Phania matricarioides* (Spreng.) Griseb. Molecules. 24(8), 1615. https://doi:10.3390/molecules24081615

Haas, J., Barbato, A., Behringer, D., Studer, G., Roth, S., Bertoni, M., Mostaguir, K., Gumienny, R., Schwede, T., 2018. Continuous Automated Model Evaluation (CAMEO) complementing the critical assessment of structure prediction in CASP12. Proteins. 86, 387-398. https://doi: 10.1002/prot.25431.

Hu, W., Li, C., Dai, J., Cui, H., & Lin, L., 2019. Antibacterial activity and mechanism of *Litsea cubeba* essential oil against methicillin-resistant *Staphylococcus aureus (MRSA).* Ind. Crops Prod. 130, 34–41. https://doi:10.1016/j.indcrop.2018.12.078

J. Mol. Graph. Model. 17, 57–61 PMID: 10660911.

Kalumbi, M. H., 2019. Effect of Vancomycin, tetracycline, *Persia americana* leaf extract and combinations thereof on antibacterial activity against pathogenic organisms. J. Pharmacogn. Phytochem. 8(1), 2733-2737.

Karaman, İ., Şahin, F., Güllüce, M., Öğütçü, H., Şengül, M., Adigüzel, A., 2003. Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. J. Ethnopharmacol. 85, 231-235.

Kohanski, M.A., Dwyer, D.J., Collins, J.J., 2010. How antibiotics kill bacteria: from targets to networks. Nat. Rev. Microb. 8(6), 423.

Krishnamoorthy, R., Athinarayanan, J., Periyasamy, V.S., Adisa, A.R., Al-Shuniaber, M.A., Gassem, M.A., Alshatwi, A.A., 2018. Antimicrobial activity of nanoemulsion on drug-resistant bacterial pathogens, Microb. Pathog. https://doi:10.1016/j.micpath.2018.04.035.

Lennet, E.H. et al., eds., 1985. Manual of clinical microbiology, 4th ed. Washington, DC, American Society for Microbiology.

Lee, S.Y., Kim, K.B.W.R., Lim, S.I., Ahn, D.H., 2014. Antibacterial mechanism of *Myagropsis myagroides* extract on *Listeria monocytogenes.* Food Control, 42, 23–28*.* https://doi:10.1016/j.foodcont.2014.01.030

Lin, L., Mao, X., Sun, Y., & Cui, H., 2018. Antibacterial mechanism of *artemisinin* / beta-cyclodextrins against methicillin-resistant *Staphylococcus aureus* (MRSA). Microbial Pathogen. 118, 66–73. https://doi:10.1016/j.micpath.2018.03.014

Lourenço, A.M., Haddi, K., Ribeiro, B.M., Corrêia, R.F., Tomé, H.V., Santos-Amaya, O., Pereira, E.J., Guedes, R.N., Santos, G.R., Oliveira, E.E., Aguiar, R.W.S., 2018. Essential oil of *Siparuna guianensis* as an alternative tool for improved lepidopteran control and resistance management practices. Sci. Rep. 8,7215. https://doi: 10.1038/s41598-018-25721-0

Mighri, H., Hajlaoui, H., Akrout, A., Najjaa, H., Neffati, M., 2010. Antimicrobial and antioxidant activities of *Artemisia herba-alba* essential oil cultivated in Tunisian arid zone. C. R. Chimie. 13, 380–386.

Nocchetti, M., Donnadio, A., Ambrogi, V., Andreani, P., Bastianini, M., Pietrella, D., Latterini, L., 2013. Ag/agcl nanoparticle decorated layered double hydroxides: synthesis, characterization and antimicrobial properties. J. Mater. Chem. B. 1, 2383–2393. https://doi.org/10.1039/C3TB00561E.

Oliveira, M. S., Cruz, J. N., Mitre, G. P., Costa, W. A., Kataoka, M. S. S., Silva, S. G., Alves, A. C. B. A., Pinheiro, J. J. V., Silva, S. E. M., Menezes, S. A. F., Menezes, T. O. A., Neto, A. M. J. C., Junior, R. N. C., 2019. Antimicrobial, cytotoxic activity of the *Syzygium aromaticum* essential oil, molecular docking and dynamics molecular studies of its major chemical constituent. J. Comp. Theor. Nanosci. 16(2), 355-364. https://doi.org/10.1166/jctn.2019.8108

Omelon, S., Georgiou, J., Habraken, W., 2016. A cautionary (spectral) tail: red-shifted fluorescence by dapi-dapi interactions. Biochem. Soc. Trans. 44, 46–49.

OriginLab. OriginPro 8, Version *8.0724*. OriginLab Corporation, Northampton, MA, USA.

Ostrosky, Elissa A. et al., 2008. Métodos para avaliação da atividade antimicrobiana e determinação da Concentração Mínima Inibitória (CMI) de plantas medicinais. Rev. bras. farmacogn. 18(2) 301-307. http://dx.doi.org/10.1590/S0102-695X2008000200026.

Oyedeji, O. A., & Afolayan, A. J., 2005. Chemical composition and antibacterial activity of the essential oil of *Centella asiatica.* Growing in South Africa. Pharm. Biol. 43(3), 249–252. https://doi:10.1080/13880200590928843

Peyret, M., Carret, G., Carre, C., Fardel, G., Flandrois, J.P., 1990. Mathematical study of the sensitivity curves of *Escherichia coli* exposed to polymyxins. Pathol. Biol. 38, 441-445.

Probst, I.S. 2012. Atividade antibacteriana de óleos essenciais e avaliação de potencial sinérgico. 112f. Dissertação (mestrado) – Universidade Estadual Paulista, Instituto de Biociências de Botucatu.

Rahman, A. A. A., Mohamed, S. M., Samy, E. M., Sayed, M. .A., Youssef, E. M. K, Hetta, H.F., 2018. Novel Formulation and Clinical Evaluation of Levofloxacin Hemihydrategel for the Treatment of Impetigo. J. Am. Sci. 14(10), 47-57. https://doi:10.7537/marsjas141018.06.

Ramachandran, G. N., Sasisekharan, V., 1968. Conformation of polypeptides and proteins. Adv. Protein. Chem. 23, 283-437.

Renner, S. S., Hausner, G., 2005. Monograph of Siparunaceae. Flora Neotropica 95. 1-256.

Robinson, A., van Oijen, A. M., 2013. Bacterial replication, transcription and translation: mechanistic insights from single-molecule biochemical studies. Nat. Rev. Microb. 11(5), 303-315. https://doi.org/10.1038/nrmicro2994.

Roosild,T.P.,Castronovo,S.,Healy,J.,Miller,S.,Pliotas,C.,Rasmussen,T.,Bartlett, ., Conway,S.J.,Booth,I.R., 2010. Mechanism of ligand-gated potassium efflux in bacterial pathogens. Proc. Natl. Acad. Sci. 107,19784–19789.

Runyoro, D., Ngassapa, O., Vagionas, K., Aligiannis, N., Graikou, K., & Chinou, I., 2010. Chemical composition and antimicrobial activity of the essential oils of four *Ocimum* species growing in Tanzania. Food Chem. 119(1), 311–316. https://doi:10.1016/j.foodchem.2009.06.028

Sanner, M.F., 1999. Python: a programming language for software integration and development.

Sanyal, G., Doig, P., 2012. Bacterial DNA replication enzymes as targets for antibacterial drug discovery. Expert opinion on drug discovery. 7(4), 327-39.

Sbayou, H., Ababou, B., Boukachabine, K., Manresa, A., Zerouali, K., Amghar, S., 2014.Chemical composition and antibacterial activity of *Artemisia herba-alba* and *Mentha pulegium* essential oils. J. Life Sci. 8, 35–41.

Schrodinger, L.L.C., 2018. The PyMOL Molecular Graphics System, Version 2.0.

Shityakov, S., Förster, C., 2014. In silico predictive model to determine vector-mediated transport properties for the blood-brain barrier choline transporter. Adv. Appl. Bioinform. Chem. 7, 23–36. https://doi:10.2147/AABC.S63749

Skočibušić, M., Bezić, N., Dunkić, V., Radonić, A., 2004. Antibacterial activity of *Achillea clavennae* essential oil against respiratory tract pathogens. *Fitoterapia*. 75(7-8), 733–736. https://doi: 10.1016/j.fitote.2004.05.009.

Steer, A.C., Danchin, M.H., Carapetis, J.R., 2007.Group A streptococcal infections in children. J. paediatr. child health. 43(4), 203-213.

Swamy, M.K., Akhtar, M.S., Sinniah, U.R., 2016. Antimicrobial properties of plant essential oils against human pathogens and their mode of action: An updated review. Evid. Based Complement. Alternat. Med. 3012462. https://doi:10.1155/2016/3012462

Tao, C., Wang, Y., Zhang, X., Li, L., Wu, Y., Han, X., Jiang, X., Lv, Z., 2019. Mechanism of Action of Essential Oils Extracted from bamboo (*Phyllostachys heterocycla* cv. *pubescens*) leaves: Chemical composition and antimicrobial activity against four food-related microorganisms. Bioresour. 14(1), 1419-1434.

Trott, O., Olson, A.J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. J. Comput.Chem. 31, 455–461. https://doi.org/10.1002/jcc.21334.

Utchariyakiat, I., Surassmo, S., Jaturanpinyo, M., Khuntayaporn, P., Chomnawang, M.T., 2016. Efficacy of cinnamon bark oil and cinnamaldehyde on antimultidrug resistant *Pseudomonas aeruginosa* and the synergistic effects in combination with other antimicrobial agents. BMC Complement. Alternat.Med.16, 158.

Valentini, C.M.A., Rodriguez-ortiz, C.E., Coelho, M.F.B., 2010. *Siparuna guianensis* Aublet (negramina): uma Revisão. Rev. Bras. Plantas Med. 12, 96–104.

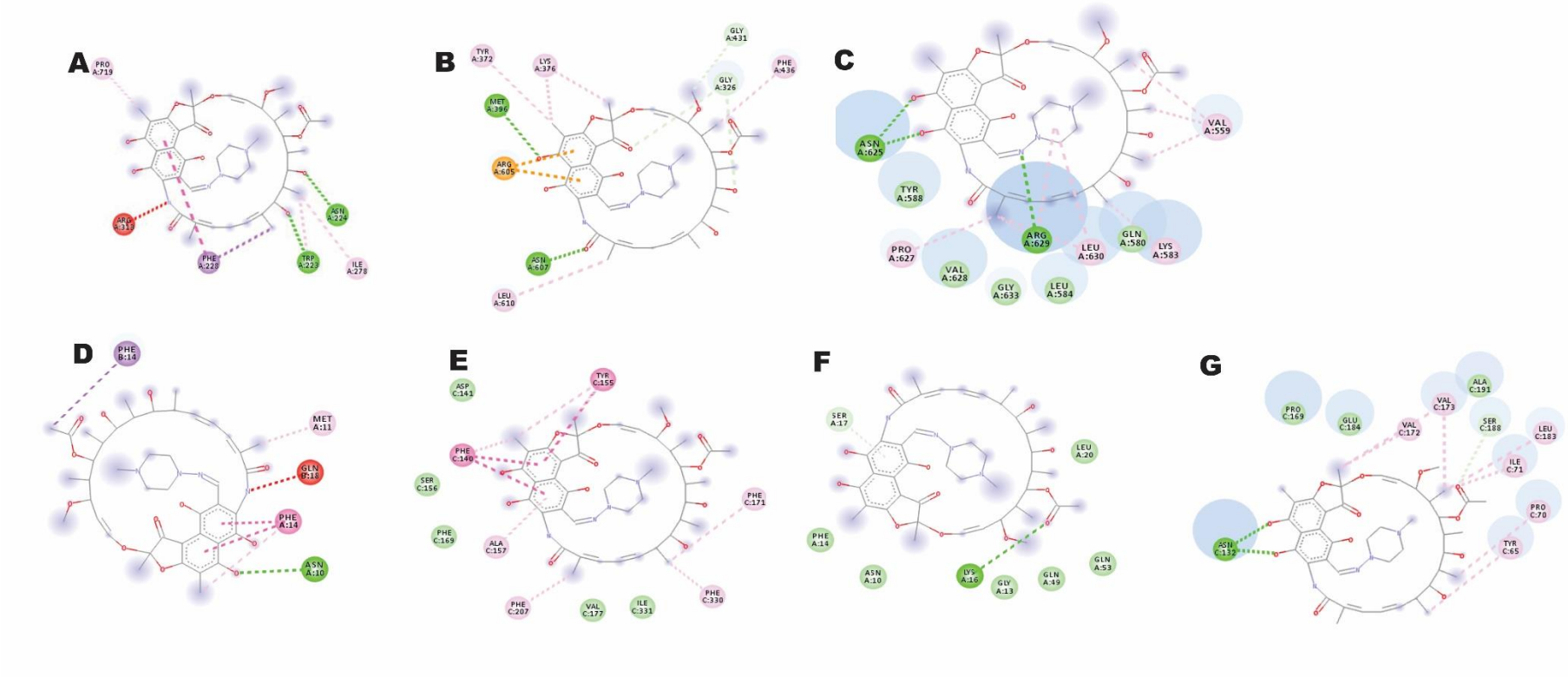
Vieira, I. C. G., Silva, J. M. C., Toledo, P. M., 2005. Estratégias para evitar a perda da biodiversidade na Amazônia. Estudos Avançados. 19(54), 153-164.

Wang, Y., Zhang, Q., Jin, Y., 2019. Epidemiology and antimicrobial susceptibility profiles of extended-spectrum beta-lactamase–producing *Klebsiella pneumoniae* and *Escherichia coli* in China. Braz. J. Microb. https://doi.org/10.1007/s42770-019-00081-7

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T., 2018. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic. Acids Res. 46(W1), W296-W303.

Xiang, Z., 2006. Advances in Homology Protein Structure Modeling. Curr. Protein. Pept. Sci. 7(3), 217–227.

Xu, C., Li, G-y, Zhang, H., Zu, Y-n, Li, Z-h, Chen J-l, 2018. Antibacterial activity of *Artemisia asiatica* essential oil against some common respiratory infection causing bacterial strains and its mechanism of action in *Haemophilus influenzae*. Microbial. Pathogen. https://doi: 10.1016/j.micpath.2017.12.032



Supplementary Figure 1. Rifamycin SV sodium 2D maps of molecular interactions with amino acids in DNA Polymerase Active site (A, B, C) and RNA Polymerase (D, E, F, G) of *E. coli* (A ,D)*, P. aeruginosa* (B, E), *S. aureus* (C, F) and *S.pyogenes* (G).

CHAPTER II - Cassava starch-based essential oil microparticles preparations: Functionalities in mosquito control and selectivity against non-target organisms

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Abstract

Due to the production facilities and great functionalities, the starch extracted from Cassava plants' (*Manihot esculenta* Crantz) roots is one of the most abundant and inexpensive raw materials used in food- and non-food industries. The utilization of starches to encapsulate plant essential oils is a relevant advance in the control of insect pests, including mosquitoes that transmit human diseases. The starch-based microencapsulation of essential oils reduces the degradation and volatilization of active components, providing more sustainable and environmentally friendly activities. Here, we investigated the potential of cassava-based starch microparticle preparations containing the essential oil of a Neotropical plant (*Siparuna guianensis* Aublet) to control larvae of *Aedes aegypti* and *Culex quinquefasciatus*. Moreover, the selectivity of the most efficient microparticles preparation was evaluated on zebrafish embryos (*Danio rerio*), an aquatic non-target organism. The characterization of encapsulated microparticles was achieved by using scanning electron microscopy (SEM), infrared spectroscopy with Fourier transform (FTIR), and thermogravimetric analysis (TGA). Our results revealed an encapsulation efficiency of 82.8 % to 95.3 %, with an average particle diameter of 8.56 □m. Cassava starch microencapsulation reduced the essential oil degradation and enhanced (up to 8 days) the persistent lethal activities (over 50 %) against both species' mosquito larvae compared to the pure essential oil. Furthermore, the exposure of aquatic non-target organisms (embryos of *D. rerio*) revealed these microparticles' adequate selectivity. Collectively, our findings demonstrate that cassava starch-based microparticles exhibit promising functionality as carriers for essential oils with mosquitocidal activities.

Keywords: food-based carriers; *Siparuna guianensis*; plant-based biorational insecticides; mosquito control; non-target toxicity.

1 INTRODUCTION

Cassava, *Manihot esculenta* Crantz, is one of the most important food crops in the world, with a production of over 290 million tons in 2020 (Cuenca et al., 2020; Tapibban et al., 2020). With roots containing high starch, cassava is widely used both in human and animal food as well as for non-food purposes in various technological and industrial products (Liu et

al., 2019). Its importance in the food industry and its worldwide distribution make the cassava plant a good starch source for encapsulation purposes (Tappiban et al., 2019).

The encapsulation technique has been studied and used to improve some materials' performance through the protection and controlled release of active ingredients (Zaitoon et al., 2021; Annunziata et al., 2020; Alarcón-Alarcón et al., 2018; Tomazelli et al., 2018). Among the polymers of interest, plant starch is an abundant and cheap natural raw material with promising potential for microencapsulation (Estevez-Areco et al., 2020; Moura & Ascheri, 2018). In addition to its excellent biodegradation capacity, starch can develop porous spherical aggregates with high encapsulation efficiency. Moreover, starch may provide wall material for microcapsule production to replace high-cost encapsulating agents (Ribeiro & Veloso, 2021; Márquez-Gómez et al., 2017).

The application of encapsulation techniques for delivering particles of plant-based essential oils is a significant advance in the control of not only agricultural insect pests but also insects that transmit human diseases (e.g., mosquitoes). There is an increasing need to develop alternatives to control mosquitoes that are not susceptible to conventional insecticides. Plants have been intensively screened for active products (such as extracts and essential oils) that have insecticidal properties capable of replacing conventional insecticides or being integrated into the management of insect pests (Benelli et al. 2021; Haddi et al., 2020; Isman 2020; Lucia et al., 2020; Rizzo et al., 2020; Ferreira et al., 2019; Borges et al., 2019; Aguiar et al., 2015). However, some essential oil physicochemical properties (e.g., volatility and miscibility in water) can compromise their persistence in the environment, requiring excessive applications (Benelli et al., 2020; Fuentes et al., 2020; Pavela et al., 2020; Mossa et al., 2017; Pavela, 2015; Asbahani et al., 2015). Indeed, high volatility and low water immiscibility reduce the larvicidal virulence of essential oils when they are directly applied in water bodies. Therefore, we aimed to develop novel strategies based on microencapsulation to increase the residual activity of these alternative bioinsecticides (Fernandes et al., 2014).

Among the promising plants for essential oil production, the aromatic and medicinal Neotropical plant (*Siparuna guianensis* Aublet) has proven potential in the control of mosquito vectors of human diseases (Ferreira et al., 2019; Aguiar et al., 2015), agricultural pests (Toledo et al., 2019; Lourenço et al., 2018; Ferreira et al., 2017a), and the control of undesired bacteria and fungi (Moura et al., 2020; Oliveira et al., 2020). The combined use of products based on *S. guianensis* essential oils and starches should be extensively studied due to their non-toxicity to humans, animals, and the environment that greatly reduces the impact of insecticides. Furthermore, recent investigations (Ferreira et al., 2019; Kumar et al., 2016) on encapsulated particle preparations used to control mosquito larvae have indicated that these novel strategies do not show any detrimental effects on the aquatic non-target predators of mosquito larvae. However, as observed for other plant-based biorational approaches (Haddi et al. 2020), such practices can not be overlooked in their potential unintended ecotoxicological effects.

Considering that zebrafish (*Danio rerio*) is an excellent animal model for detecting safe levels of environmental contaminants (Bailone et al., 2019) and for human and animal health (Capriello et al., 2020; Chen et al., 2012), investigations that evaluate the detrimental or stimulatory sublethal effects of essential oil starch-based encapsulated preparations on these organisms would allow significant advances in the assessment of the potential ecotoxicological risks for such novel insecticidal alternatives. Here, we encapsulated *S. guianensis* essential oil microparticles using cassava starch and verified whether starch-based encapsulation retained for more extended periods the action of *S. guianensis* essential oil against mosquito (i.e., *Aedes aegypti* and *Culex quinquefasciatus*) larvae. Finally, we assessed the prepared *S. guianensis* microparticles' selectivity to the non-target zebrafish embryos as an indicator of potential toxicity to human and animal health.

2 MATERIALS AND METHODS

2.1 Plant material and essential oil extraction

Leaves of *S. guianensis* were collected between February and May 2019 from trees located in the municipality of Gurupi-Brazil (11°43'45" S, 49°04'07" W) and transported for essential oil extraction according to the methods described in Ferreira et al. (2017b). The taxonomic identification of the collected specimens was performed by herbarium specialists from the Federal University of Tocantins (Porto Nacional-TO, Brazil - 10,496). The present investigation was registered in Brazil – SISGEN under number A7CAD12. We extracted the essential oil from the collected *S. guianensis* leaves using the steam distillation method with a Clevenger apparatus as described in Moura et al. (2020). Briefly, we crushed 300 g of fresh *S. guianensis* leaves and mixed the powder with 1000 mL of distilled water. The mixture was placed in a 2000 mL round-bottom flask coupled to Clevenger apparatus. The round-bottom flask mixture was heated, and the vapors produced passed through the condensation column with a cooling system. The condensed essential oil was collected, cooled, and stored in the fridge.

2.2 Mosquito and zebrafish populations

The 3rd instar larvae of *A. aegypti* and *C. quinquefasciatus* used in the experiments were obtained from colonies originally established from field-collected insects from regions where no insecticides have been used for the control of mosquitoes (Aguiar et al., 2015) in the state of Tocantins, Brazil (11°40'55.7″ latitude S, 49°04'3.9" longitude W). The colonies were maintained under controlled laboratory conditions (T°: 26 °C; RH: 70%) in the Integrated Pest Management (IPM) laboratory of the Universidade Federal do Tocantins (UFT).

The zebrafish (*D. rerio*) embryos used in the toxicological assays were supplied by the Toxicological Genetics laboratory of the Universidade de Brasilia (UnB). The adult fishes were kept in an automated water recirculating system and provided with water filtered through

activated carbon and aerated to eliminate chlorine (ZebTec, Tecniplast, Italy). The physical and chemical characteristics of the system were maintained with pH 7.2 - 7.6, hardness 6.7 °dH, temperature 26 ± 1 °C, and conductivity 728 µS. The aquarium room has a photoperiod of 12 h of light and 12 h of darkness. The fish are fed two to three times a day with a commercial food (SERAVipan ©; Tetramin ©) and live brine shrimps (*Artemia salina* nauplii).

2.3 Chemical analysis of the *S. guianensis* essential oil

The essential oil's chemical composition was assessed and identified three times by gas chromatography (GC-FID) using a Chemito 8510 GC instrument (Chemito Technologies Ltd, Mumbai, India Pvt.). Separation of the major constituents was performed using a capillary column BP-5 (30 × 0.53 mm i.d., 1.0 mm film thickness), and hydrogen was used as the drag gas at a flow rate of 5 mL/min and a pressure of 20 psi. The GC oven temperature was programmed at 70 to 210 °C with a 2.5 °C/min heating ramp with the injector and detector temperatures (FID) maintained at 230 °C. The GC-MS analysis was performed on a DSQ MS (Thermo Electron Corporation, Waltham, MA, USA) using a BP-5 (30 × 0.25 × 0.25 mm) capillary column. Helium was used as the drag gas at a 1 mL/min flow rate with a 1:20 split. The column's temperature was programmed to range from 65 to 210 °C using a heating ramp at a rate of 3 °C/min. Mass spectra were obtained in the range of 40 to 650 amu, operating at 70 V, and the source was maintained at 200 °C following Ferreira et al. (2017b).

2.4 *S. guianensis* essential oil microparticles preparation

The cassava starch (*M. esculenta*) product (Yoki, General Mills Inc. Yoki, São Bernardo Do Campo, SP**,** Brazil) used in the microencapsulation process was purchased locally in supermarkets. This starch has a carbohydrate content of 85 %, 12 % humidity, and 3 % other constituents (minerals) with a gelatinization temperature between 58 and 70 °C, as described by Vicentini (2003). The microencapsulation consisted of heating 2 g of starch in 30 mL of water to its gelatinization temperature (68 °C). Then polysorbate was added at a ratio of 2 % with dropwise addition of *S. guianensis* essential oil under constant stirring, and finally, the preparation was cooled to room temperature (Collins et al., 2019). The resulting emulsion was lyophilized by freezing at -20 °C immediately after preparation. After 24 h, the frozen emulsion was dried for longer than 48 h at -45 °C under a pressure of less than 0.120 mbar using a freeze-drier as described by Samakradhamrongthai et al. (2015) with minor adaptations. We varied the proportions of *S. guianensis* essential oil (O) and starch cassava (S) to create the following samples (O:S): OS1:1, OS1:2, OS1:3, and 0:1 as a control (SG) sample containing only gelatinized starch and polysorbate.

2.5 Morphological and thermogravimetric characterization of *S. guianensis* essential oil microparticles

The morphology and particle size of the dried microparticles were determined by scanning electron microscopy (SEM) (Ferreira et al., 2019). The lyophilized sample was covered with gold for 180 s using an Emitech K550 (Emitech, Kent, UK) and observed on an SEM Zeiss DSM 962 (Carl Zeiss, Oberkochen, Germany), at 15 kV. The number and mean diameter (with standard deviation) of the microparticles were determined for 400 observations using the software ImageJ (National Institutes of Health, NIH, Maryland, USA). The characterization of microparticles by Fourier-transform infrared spectroscopy (FTIR) spectra was obtained using an IRAffinity^{-1} FTIR (Shimadzu, Kyoto, Japan) coupled to a HATR MIRacle module with a ZnSe prism (Pike technologies, Madison, USA) using 32 scans at a resolution of 4 cm^{-1} from 4000 to 700 cm^{-1}.

The thermogravimetric analysis (TGA) was performed using a TGA-50 instrument (Shimadzu, Kyoto, Japan). The samples were heated at a constant rate of 10 °C min^{-1} in the temperature range of 25 to 900 °C under nitrogen flow. The starch/essential oil percentage was estimated from the fraction of the dTG area related to the second stage of mass loss followed by an interpolation of the results (Equation 1).

$$\frac{(fOS - fEO)}{Xos} = \frac{fSG - fEO}{100\%} \qquad (1)$$

where:

fSG, fEO, and fOS are the mass fractions of the second peak of degradation in SG, essential oils (EO), and OS samples minus the masses of the water peaks in each analysis, respectively. Xos is equal to the estimated starch fraction in the oil microparticle sample.

The encapsulation efficiency (EE) was estimated using Equation 2.

$$EE = \left(\frac{Mass\ of\ loaded\ EO}{Mass\ of\ initial\ EO}\right) X\ 100 \qquad (2)$$

2.6 Toxicity bioassays

Third-instar larvae of *A. aegypti* and *C. quinquefasciatus* were used to assess the lethal activity of the *S. guianensis* essential oil microparticles encapsulated in cassava starch. Twenty-five larvae were added to a container containing 30 cm³ of essential oil microparticles solution or a control solution. After a 24 h exposure period, larval mortality was assessed, and all larvae (dead and alive) were removed from the solution. At this time, another group of 25 live larvae was placed into the same solution. This operation was repeated every 24 h until no mortality was observed. The tested solutions consisted of starch microparticles containing *S. guianensi*s in three proportions (OS1:1, OS1:2, and OS1:3), an SG control (containing only gelatinized starch and polysorbate), and pure *S. guianensis* essential oil. The tests were

performed in four different concentrations as proposed by Aguiar et al. (2015): 0.167, 0.500, 0.834, and 1.667 mg of microparticles /cm³ of solution.

2.7 Selectivity bioassays

To evaluate the selectivity of *S. guianensis* essential oil microparticles against aquatic non-target organisms, we evaluated lethal (embryo mortality) and sublethal (hatching embryos, cardiac edema, altered yolk sac, and behavioral effects) effects of this alternative insecticide on zebrafish embryos. Our toxicological assays were based on the Fish Embryo Toxicity (FET) tests suggested by the Organization for Economic Co-operation and Development (OECD) toxicity assessment protocols (OECD N° 236, 2013). After collecting the embryos from the aquariums, they were washed and immediately distributed into microplates with the test solutions, allowing them to begin the exposure in the initial embryonic stages. The exposure was performed in 96-well microplates with 200 µL of each concentration. The tests were conducted in a climatic chamber with conditions identical to the cultivation room. The test solutions were prepared with water samples with the same physical and chemical characteristics as those used to cultivate the adult zebrafish. We tested the essential oil microparticle concentrations of 0 (i.e., negative control, with only water); 0.100; 0.171; 0.295; 0.507; 0.807; and 1.500 mg/cm^3 (mg of microparticle/cm³ of water). For each essential oil microparticle concentration, the tests were performed in triplicate, totalizing 60 organisms. The exposure time was 96 h. We only used the microparticle with an OS1:3 ratio as these microparticles exhibited the highest toxicity for mosquito larvae in the larvicidal bioassays (see the results section for more details).

2.8 Statistical analysis

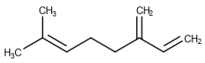
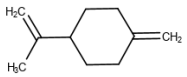
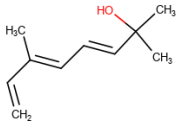
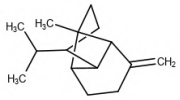
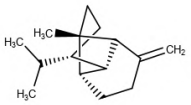
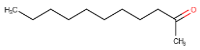
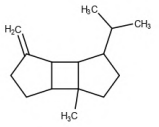
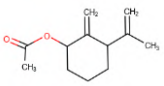
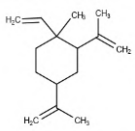
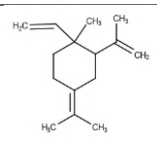
A mortality over time graph for *A. aegypti* and *C. quinquefasciatus* with the different formulations was plotted using nonlinear regression parameters determined by OriginPro® 8 software. The lethal concentrations (LCs) and effect concentration (EC) of the zebrafish data were determined by probit analysis (Finney, 1971) using POLO PLUS statistical software (Leora Software Berkeley, CA, USA). For the analysis of non-target organisms, the statistical package Sigma Plot 12.5 was used. Unidirectional ANOVA was used to detect differences between groups for normally distributed datasets. When the data did not pass the Kolmogorov-Smirnov normality test, the Levene homogeneity test of variance, Kruskal-Wallis, was used. The Dunnett's test (for parametric analysis) or Dunn' test (for non-parametric analysis) were used to detect significant differences between the tested concentrations and the control. In all analyses, a probability value of less than 0.05 was considered statistically significant ($P < 0.05$).

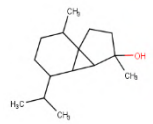
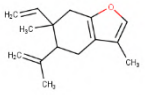
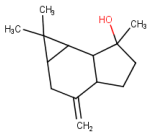
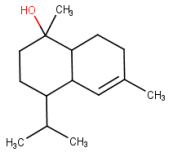
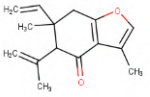
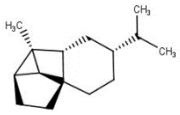
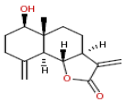
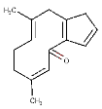
3 RESULTS

3.1 Chemical analysis of *S. guianensis* essential oil

Chemical characterization revealed 18 different constituents in the extracted *S. guianensis* essential oil, with the major components being the monoterpene β-myrcene (39.16 %), sesquiterpenes epicurzerenone (16.02 %), and β-copaene (9.33 %) (Table 1).

Table 1 – Chemical composition, concentrations (%) and Kovats index for the *S. guianensis* essential oil

Compound	Chemical structure	%	RI[a]	RI Lit.[b]
β-Myrcene		39.16	958	988
Pseudolimonene		0.95	1013	1005
2,6-Dimethyl-3,5,7-octatriene-2-ol, Z,Z-		1.14	1090	1087
β-ylangene		1.64	1216	1272
β-copaene		9.33	1216	1220
2-Undecanone		6.25	1251	1294
β-Bourbonene		0.76	1339	1387
Cyclohexanol, 2-methylene-3-(1-methylethenyl)-, acetate, cis-		2.30	1341	1347
β-elemene		1.99	1398	1389
Elixene		5.88	1431	1492

Epi-cubebol		1.59	1498	1493
Isofuranogermacrene		3.91	1532	1510
Spathulenol		2.73	1536	1577
α-Cadinol		0.86	1580	1602
Epicurzerenone		16.02	1611	1605
Germacrene B		3.97	1613	1591
Reynosin		0.72	1741	1752
Isofuranodienone		0.79	1808	1814
		99.99		

[a] Retention index experimental; [b] Retention index literature (Adams, 2007; NIST, 2018).

3.2 Characterization of *S. guianensis* essential oil microparticles encapsulated with starch cassava

Scanning electron microscopy (SEM) analysis showed that the *S. guianensis* essential oil microparticles presented a smoothly rounded and integral appearance without any surface holes (Figure 1A). The average size of the particles ranged from 0.6 to 24 μm, with an average diameter of 8.56 ± 5 μm (Figure 1B).

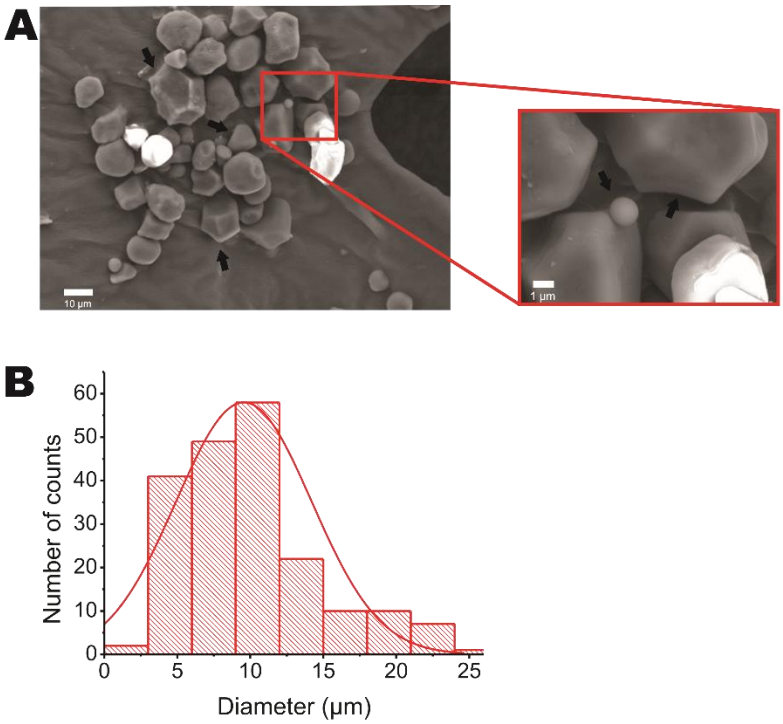


Figure 1 - (A) SEM image (with an approximation of 850x) of OS1:1 cassava starch-encapsulated microparticles of *S. guianensis* essential oil. Arrows indicate irregular appearance, forms rounded and integrals, the surface without holes. (B) The size distribution of *S. guianensis* essential oil microparticles (*n* = 400) recorded in SEM images. The mean diameter was 8.56 (± 5, standard deviation) µm.

These microparticles presented broadband between 3600 and 3200 cm^{-1}, characteristic of the hydroxyl functional group, and a band at 3080 cm^{-1}, attributed to the stretching vibration of CH from olefins (Figure 2). Furthermore, while peaks at 2958, 2926, and 2857 cm^{-1} refer to elongation vibrations, peaks at 1447 and 1377 cm^{-1} refer to angular deformations of C-H bonds of aliphatic chains, respectively (Figure 2). The C = C double bonds' absorption peaks present in the unsaturated fatty acids were observed at 1603 and 990-800 cm^{-1}. The absorption at 1799 cm^{-1} is characteristic of the ester functional group (Figure 2).

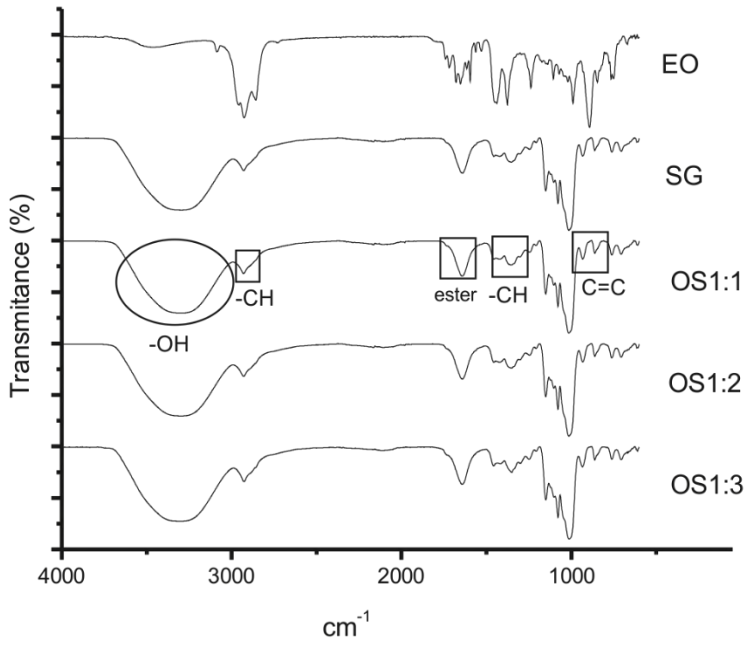


Figure 2 – FTIR spectra of pure *S. guianensis* essential oil and microparticles OS1:1, OS1:2, OS1:3, and SG (control).

TGA analysis presented the thermal properties of *S. guianensis* essential oil microparticles and revealed that the calculated encapsulation efficiency was 82.8 %, 84.8 %, and 95.3 % for the particles of OS1:1, OS1:2, and OS1:3, respectively (Figure 3).

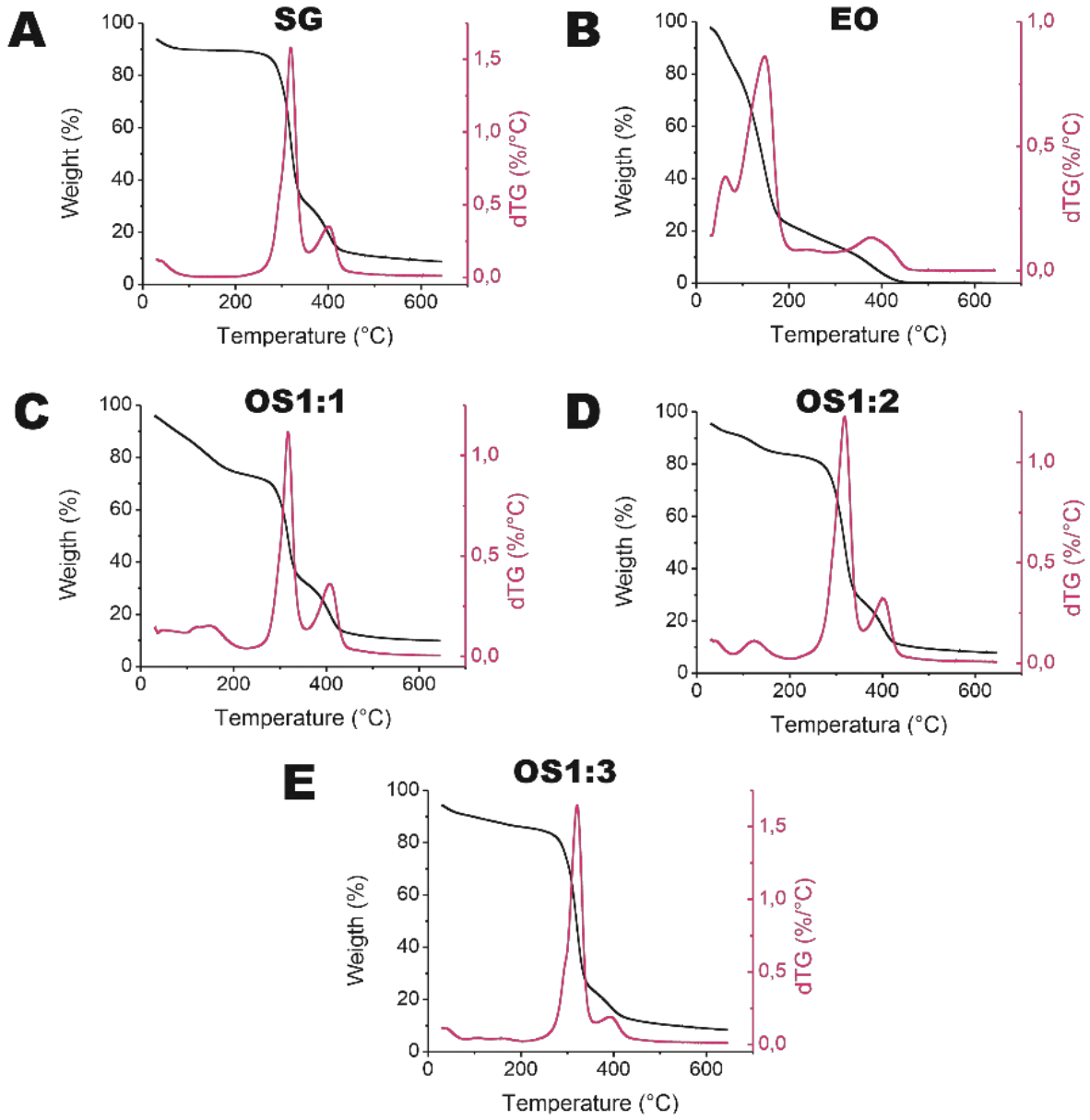


Figure 3 – TGA curves of microparticles. (A) SG (control), (B) pure *S. guianensis* essential oil, (C) OS 1:1, (D) OS1:2, (E) OS1:3. Black lines represent the residual weight, and magenta lines represent the dTG.

3.3 Bioassays

Bioassays with *S. guianensis* essential oil microparticles and pure *S. guianensis* essential oil were also evaluated. The toxicity to *A. aegypti* and *C. quinquefasciatus* were obtained for each of the concentrations (Figure 4). The curve adjustment parameters are described in Supplementary Table 1. The SG (control) particles without essential oil presented an insignificant number of deaths (less than 5 %), indicating the absence of any larvicidal activity in this compound. The *S. guianensis* essential oil microparticles OS1:1, OS1:2, and pure *S. guianensis* essential oil did not show high mortality at a concentration of 0.167 mg/cm³.

However, for the OS1:3 microparticles, there was 50 % mortality for approximately three days. This result demonstrates an efficient composition at a low concentration. The OS1:1 microparticles at a concentration of 0.834 mg/cm³ showed 50 % larvicidal activity for 8.8 and 8.3 days for *A. aegypti* and *C. quinquefasciatus*, respectively. The same mortality was observed for 9.7 days (on *A. aegypti*) and 8.7 days (effect on *C. quinquefasciatus*) when using the OS1:2 microparticles (Figure 4).

The OS1:3 microparticles were effective at causing a 50 % mortality up to 12.5 days at the same concentration (0.834 mg/cm³) for controlling *A. aegypti*. In this situation, we observed that the starch fulfills a protective function for *S. guianensis* essential oil, reducing its volatilization, and increasing larvicidal activity. At a concentration of 1.667 mg/cm³, all microparticles (including pure *S. guianensis* essential oil) caused 100 % mortality on the first test day. This result demonstrates *S. guianensis* essential oil's ability to kill the larvae of *A. aegypti* and *C. quinquefasciatus* (Figure 4).

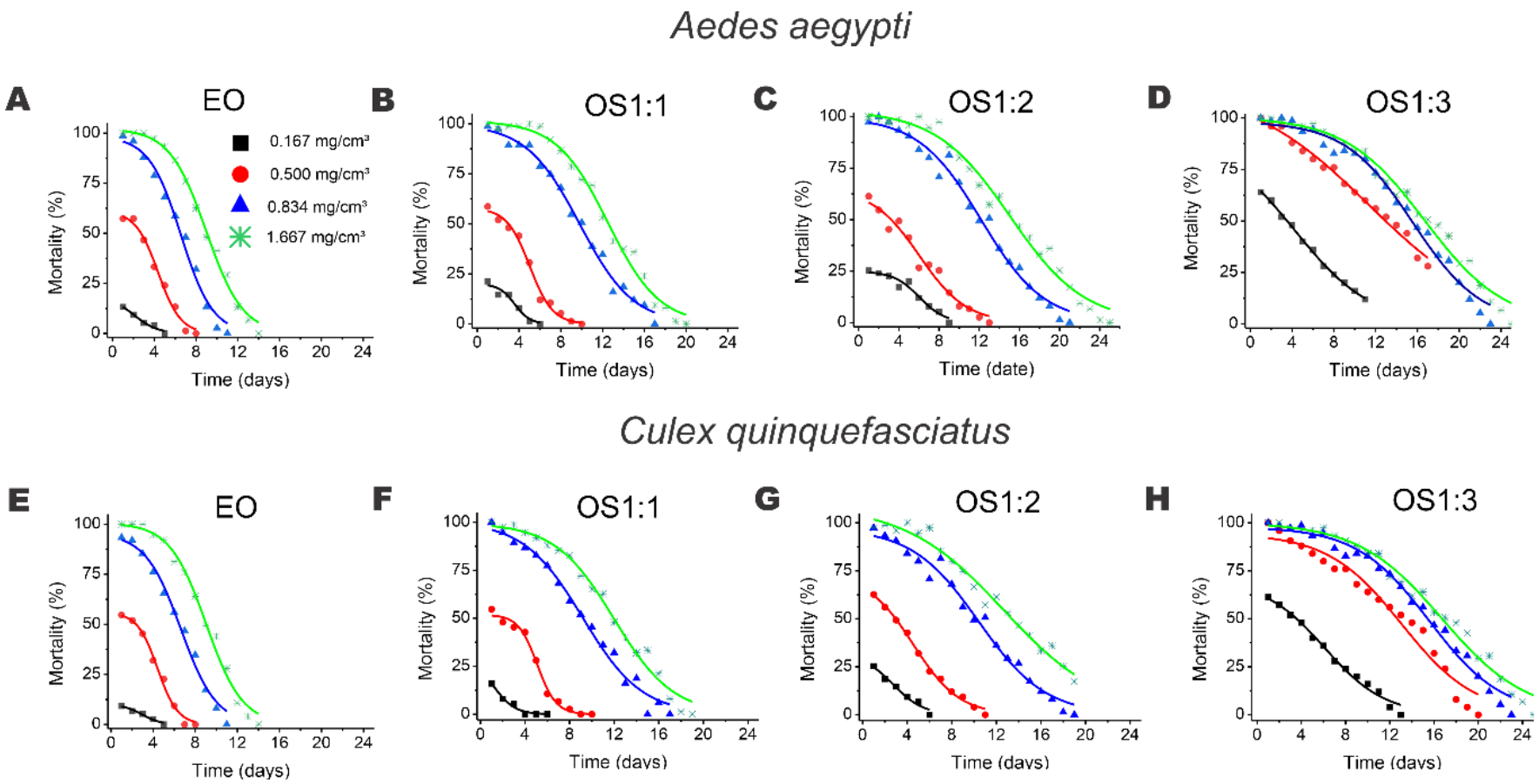


Figure 4 – Residual activities of cassava starch-encapsulated microparticles of *S. guianensis* essential oil (at different concentrations) against larvae of *A. aegypti* and *C. quinquefasciatus* mosquitoes. The parameters of the nonlinear regressions are described in Supplementary Table 1. Each symbol represents the average of three replicates.

3.4 Selectivity non-target organism

The *S. guianensis* essential oil OS1:3 proportion microparticles (i.e., the most toxic against larvae of both *A. aegypti* and *C. quinquefasciatus* mosquitoes) homogeneously diluted in all the concentrations used, and an overview of the embryotoxicity results are shown in Figure 5. After 24 h of exposure, approximately 20 % of the zebrafish embryos exposed to the highest concentration (1.500 mg/cm^3) had died. At 48 h, only organisms exposed to concentrations of 0.807 and 1.500 mg/cm^3 had died, with death rates of more than 30 % and 100 %, respectively. After 96 h, the embryos exposed to a concentration of 0.507 mg/cm^3 hatched at a rate above 50 %, and the estimated LC_{50} value was 0.936 mg/cm^3 (Figure 5). The sublethal effects were observed until the last day of exposure (96 h), and it was possible to determine EC_{50} values for altered yolk sac (0.374 mg/cm^3, Fig 6A) and cardiac edema (0.283 mg/cm^3, Fig 6B). Furthermore, exposure to essential oil-microencapsulated microparticles sublethally altered the side swimming (for 40 % of embryos exposed to 0.295 mg/cm^3) and back swimming (for 50 % of tested embryos exposed to 0.507 mg/cm^3) of zebrafish embryos (Figure 7). Behavioral changes were not observed in any of the experiments using lower concentrations.

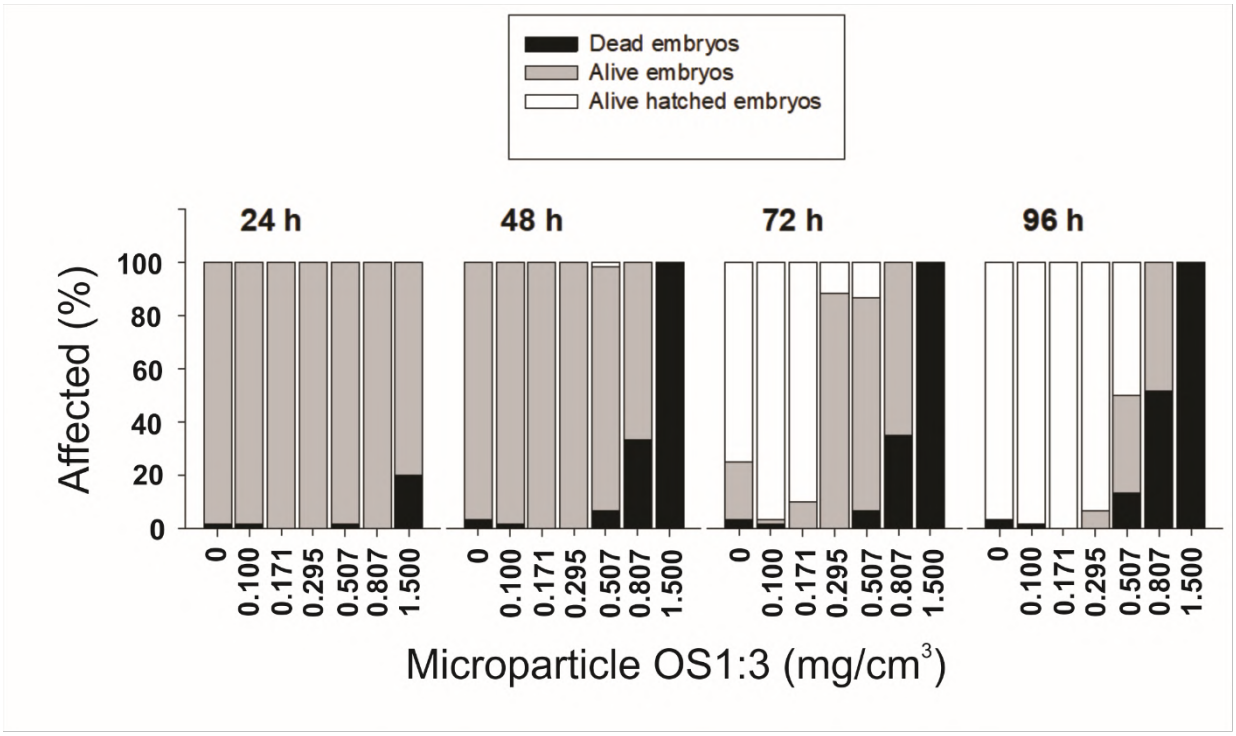


Figure 5 – Toxicity of the OS1:3 *S. guianensis* essential oil microparticle with different concentrations to zebrafish embryos.

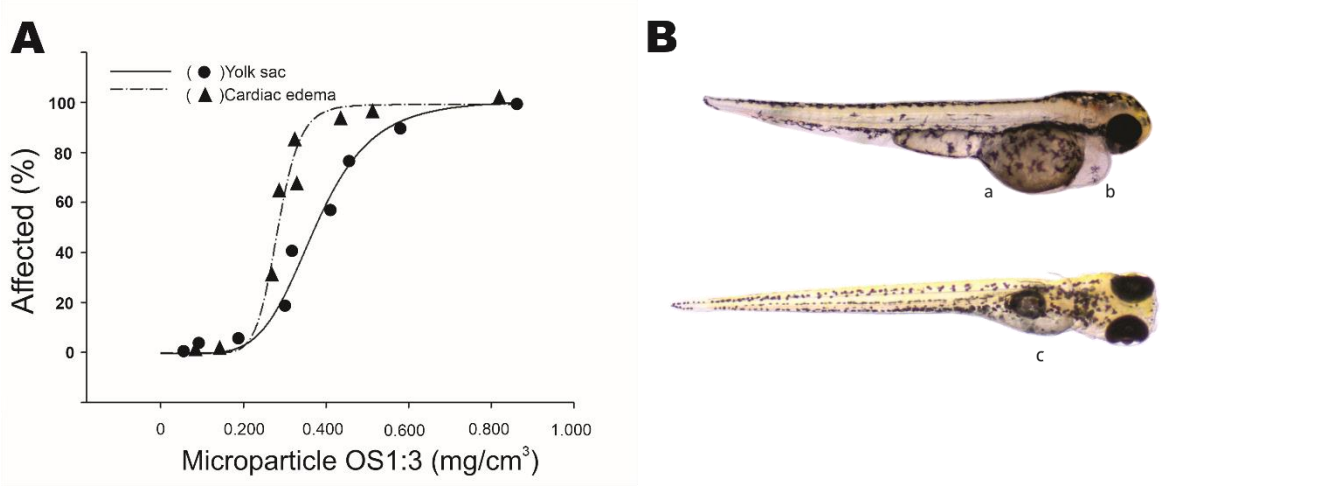


Figure 6 – (A) Percentage of zebrafish embryos with alterations on yolk sac (filled circles) or exhibiting cardiac edema (filled triangles) after 96 h of exposure to OS1:3 *S. guianensis* essential oil microparticles. Each symbol represents the average of three replicates. Lines represent the fit obtained by using the probit model for each data set. (B) Representation of a zebrafish embryo exhibiting altered yolk sac (a) and cardiac edema (b) comparatively with an embryo without alterations (c).

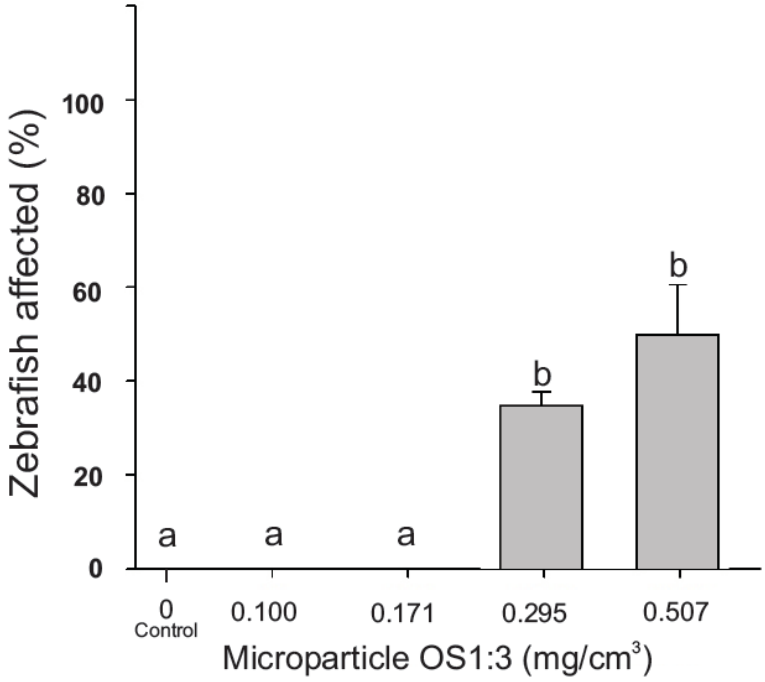


Figure 7 – Percentage of zebrafish embryos exhibiting altered swimming behaviors (side and back swimming) after the exposure (96 h) to OS1:3 *S. guianensis* essential oil microparticles. Data are presented as mean ± standard deviation (SD). Groups with different letters are significantly different ($P < 0.05$) compared to control.

4 DISCUSSION

We demonstrated cassava starch's potential to encapsulate and carry particles of the essential oil of *S. guianensis*. The use of cassava starch promoted long-lasting activities of this alternative insecticide against larvae of *A. aegypti* and *C. quinquefasciatus*. We then

determined the thermal characteristics of the microencapsulates and the microencapsulation efficiency. This research revealed that the cassava starch-based *S. guianensis* microparticles exhibited good selectivity against embryos of zebrafish *D. rerio*. This result proved that this product is safe for use in aquatic environments.

Our chromatographic analysis revealed that the major components in the *S. guianensis* essential oil are β-myrcene (39.16 %), epicurzerenone (16.02 %), and β-copaene (9.33 %). These components have been shown to be toxic to some agricultural and urban pests (Sun et al., 2020; Peterson et al., 2020), but their use as insecticides requires some adjustments because they are volatile nonpolar molecules. Therefore, we microencapsulated the *S. guianensis* essential oil with cassava starch to reduce the volatility-related losses of efficiency. The positive result of our technique was a combination of effects, including encapsulation efficiency, microparticles structure, and an interaction between the carrier (i.e., cassava starch) and the different concentrations of essential oil.

The encapsulation efficiency was determined using TGA curves. The encapsulation efficiency ranged from 82.8 % to 95.3 %, which are better results than those reported by Samakradhamrongthai et al. (2015), who obtained 66.74 % efficiency using octenyl succinic anhydride starch. Such differences in efficiency may be associated with the use of polysorbate, as this compound acts as an emulsifying agent, improving the interaction between the essential oil and the starch (Kishore et al., 2011). Therefore, we were able to record the microcapsule degradation in three stages: *1)* the evaporation of water at temperatures up to 110 °C, *2)* further degradation starting at a temperature of 279 °C with the maximum degradation at 376 °C, and *3)* the final degradation starting at 380 °C and ending at 425 °C. The first stage involved the degeneration of water molecules, causing small peaks. The second stage temperature is associated with the interaction between starch and the essential oil, as recorded by Fortunati et al. (2016), and the third stage may represent the elements with long chains and polysorbate described by Kishore et al. (2011).

At the structural level, our prepared microencapsulates were integral, and without holes on their surface, which are good indicators of stability (Murúa-Pagola et al., 2009). Their average size (8.56 μm) was smaller than the size of the microparticles similarly encapsulated with starch reported in previous studies and ranged from 30 to 40 μm (Murúa-Pagola et al. 2009; Marquéz-Gomez et al. 2017). Differences in the size of the encapsulated particles are generally influenced by the technique used, where a higher temperature may cause the fusion of two or more microparticles, thereby providing larger diameter microparticles (Glenn et al., 2010).

Furthermore, the ratio of oil to starch can reduce efficiency losses through encapsulation efficiency and the physical structure. An excess of oil could result in a rough surface, influencing microparticles' size (Marquéz-Gomez et al., 2017). The OS1:3 sample showed small differences in the FTIR results in relation to the microparticle SG (control). Such

differences were due to the lower proportion of essential oil used in its preparation, thus suffering less structure variation. As the proportion of essential oil increased in the microparticles OS1:1 and OS1:2, we observed differences in the microparticles structure. This result is in concordance with previous reports that used starch for essential oil encapsulation (Tongdeesoontorn et al., 2011), increasing the peak intensity at 934 cm^{-1} in relation to the other peaks. The bands occurring in the 1000 cm^{-1} region are related to the starch's crystalline structure, which is related to its retrogradation after the lyophilization process (Vicentini, 2003).

After verifying that the *S. guianensis* essential oil microparticles encapsulated in cassava starch possessed good stability, these preparations were used in toxicological bioassays. Their larvicidal activity was compared to the activity of pure *S. guianensis* essential oil. Our results revealed that the pure essential oil showed greater larvicidal activity in the initial days but significantly decreased over time. This result is probably due to the lower water immiscibility of the pure essential oil and its components' high volatility (Portella et al. 2014). Using an encapsulation technique to protect and slow the essential oil release has been demonstrated in previous investigations (Peres et al., 2020; González et al., 2016; Bringas-Lantigua & Pino, 2012). In our investigation, the starch layer acted as an essential oil protector and contributed to a longer larval mortality period. Our results with the OS1:3 microparticles at a concentration of 1.667 mg/cm³ showed an increase of more than 80 % in the lethal activity time (over 50 %) compared to pure *S. guianensis* essential oil.

Furthermore, the increase in the starch proportion in the microcapsules, while maintaining the same concentrations of pure *S. guianensis* essential oil, promoted an adequate level of larvicidal activity for longer periods. They effectively killed mosquito larvae for up to 24 days, as observed in the OS1:3 microparticles for both *A. aegypti* and *C. quinquefasciatus* larvae. Moreover, the OS1:3 microparticles at the lowest concentration used (0.167 mg/cm^3) killed 50 % of the larvae of *A. aegypti* (at 2.7 days) and *C. quinquefasciatus* (at 2.3 days). Therefore, our formulation performed better and for longer periods than chitosan-based microparticles containing double the active ingredient concentration (0.374 mg/cm^3) of *S. guianensis* essential oil (Ferreira et al., 2019). This result reinforces the great potential of our cassava starch-based microencapsulation technique. Our research supports previous investigations that have reported the efficiency of starch-based microencapsulation of essential oils to control insect microbial pests (Ahsaei et al., 2019; López et al., 2014; Tomazelli et al., 2018).

In addition to the prolongation of larvicidal activity, our results revealed that the *S. guianensis* essential oil microparticles also presented low toxicity to zebrafish embryos. The zebrafish embryos were selected as a non-target aquatic organism showing high genomic homology with human (> 70 %) and similarity of physiological responses, making it an attractive model organism to evaluate food toxicity and use to develop novel medicines (Haddad et al., 2019; Sanjeewa et al., 2018; Kang et al., 2013). Here, after the 96 h exposure

period and using the most efficient larvicidal cassava starch-based microparticles (i.e., OS1:3 microparticles), the LC_{50} for the zebrafish embryos was 0.936 mg/cm^3, which is far beyond the toxicity level (i.e., 0.100 mg/cm^3) preconized by the Organization for economic co-operation and development (OECD, 2013). Furthermore, even for sublethal effects (e.g., embryo hatching), the impact of the cassava starch-based *S. guianensis* essential oil microparticles could be observed only at concentrations higher than 0.507 mg/cm^3, which are more than 100-fold higher than those concentrations (0.05 mg/cm^3) recorded for investigations using *Cyperus articulates* essential oil (Brillatz et al., 2020).

5 CONCLUSION

Cassava starch demonstrated an excellent capacity to encapsulate the *S. guianensis* essential oil and produced microparticles that exhibited prolonged activity against *A. aegypti* and *C. quinquefasciatus* mosquitoes. Furthermore, these cassava starch-based *S. guianensis* essential oil microparticles presented low toxicity to non-target aquatic organisms (embryos of zebrafish). This result reinforces the potential of environmentally friendly alternatives available for mosquito control.

Acknowledgments

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Declarations of interest

None.

REFERENCES

Adams, R. 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry (fourth ed.), Allured Publishing Corp., Carol Stream, IL, USA.

Aguiar, RWS, Santos, SF, Morgado, FS, Ascencio, SD, Lopes, MM, Viana, KF, Didonet, J, Ribeiro, B M, 2015. Insecticidal and repellent activity of *Siparuna guianensis* Aubl. (negramina) against *Aedes aegypti* and *Culex quinquefasciatus*. Plos One. 10(2): e0116765. doi:10.1371/journal.pone.0116765

Ahsaei, SM, Rodríguez-Rojo, S., Salgado, M., Cocero, MJ, Jahromi, KT, Amoabediny, G., 2019. Insecticidal activity of spray dried microencapsulated essential oils of *Rosmarinus officinalis* and *Zataria multiflora* against *Tribolium confusum.* Crop Protection. 104996. doi:10.1016/j.cropro.2019.104996

Alarcón-Alarcón, C., Inostroza-Riquelme, M., Torres-Gallegos, C., Araya, C., Miranda, M., Sánchez-Caamaño, JC, Moreno-Villoslada, I., Oyarzun-Ampuero, FA, 2018. Protection of astaxanthin from photodegradation by its inclusion in hierarchically assembled nano and microstructures with potential as food. Food Hydrocoll. 83, 36-44. doi:10.1016/j.foodhyd.2018.04.033

Annunziata, G., Jiménez-García, M., Capó, X., Moranta, D., Arnone, A., Tenore, GC, Sureda, A., Tejada, S., 2020. Microencapsulation as a tool to counteract the typical low bioavailability of polyphenols in the management of diabetes. Food Chem. Toxicol. 139, 111248. https://doi.org/10.1016/j.fct.2020.111248

Asbahani, AE, Miladi, K., Badri, W., Sala, M., Addi, EHA, Casabianca, H., Mousadik, AE, Hartmann, D., Jilale, A., Renaud, FNR, Elaissari, A., 2015. Essential oils: From extraction to encapsulation. Int. J. Pharm. 483 (1–2), 220-243. https://doi.org/10.1016/j.ijpharm.2014.12.069

Bailone, RL, Aguiar, LK, Roca, RO, Borra, RC, Corrêa, T., Janke, H., Silva, HC, 2019. Zebrafish as an animal model for food safety research: trends in the animal research. Food Biotechnol. 33 (4), 283-302. DOI: 10.1080/08905436.2019.1673173

Benelli, G.; Rizzo, R.; Zeni, V.; Govigli, A.; Samkova, A.; Sinacori, M.; Lo Verde, G.; Pavela, R.; Cappellacci, L.; Petrelli, R.; Spinozzi, E.; Morshedloo, M.R.; Maggi, F.; Canale, A., 2021. *Carlina acaulis* and *Trachyspermum ammi* essential oils formulated in protein baits are highly toxic and reduce aggressiveness in the medfly, *Ceratitis capitata*. Ind. Crops Prod. 161, 113191. https://doi.org/10.1016/j.indcrop.2020.113191

Benelli, G., Pavela, R., Cianfaglione, K., Sender, J., Danuta, U., Maślanko, W., Canale, A., Barboni, L., Petrelli, R., Zeppa, L., Aguzzi, C., Maggi, F., 2020. Ascaridole-rich essential oil from marsh rosemary (*Ledum palustre*) growing in Poland exerts insecticidal activity on mosquitoes, moths and flies without serious effects on non-target organisms and human cells. Food Chem. Toxicol. 138, 111184. https://doi.org/10.1016/j.fct.2020.111184

Borges, JCM, Haddi, K., Oliveira, EE, Andrade, BS, Nascimento, VL, Melo, TS, Didonet, J., Carvalho, JCT, Cangussu, AS, Soares, IM, Ascencio, SD, Raposo, NRD, Aguiar, RWS, 2019. Mosquiticidal and repellent potential of formulations containing wood residue extracts of a Neotropical plant, *Tabebuia heptaphylla*. Ind. Crops Prod. 129, 424-433. https://doi.org/10.1016/j.indcrop.2018.12.022

Brillatz, T., Jacmin, M., Queiroz, EF, Marcourt, L., Slacanin, I., Petit, C., Carrupt, P., Bum, EN, Herrling, P., Crawford, AD, Wolfender, J., 2020. Zebrafish bioassay-guided isolation of antiseizure compounds from the Cameroonian medicinal plant *Cyperus articulatus* L.. Int. J. Phytomedicine. 70, 153175 doi: https://doi.org/10.1016/j.phymed.2020.153175

Bringas-Lantigua, M., Pino, JA, 2012. Microencapsulacion de saborizantes mediante secado por atomizacion. *Instituto De Investigaciones Para La Industria Alimentaria*. 11(2), 34–68.

Capriello, T., Visone, IM, Motta, CM, Ferrandino, I., 2020. Adverse effects of E150d on zebrafish development. Food Chem. Toxicol. 111877. https://doi.org/10.1016/j.fct.2020.111877

Chen, Y., Yang, Z., Wen, C., Chang, Y., Wang, B., Hsiao, C., Shih, T., 2012. Evaluation of the structure–activity relationship of flavonoids as antioxidants and toxicants of zebrafish larvae. Food Chem. 134 (2), 717-724. https://doi.org/10.1016/j.foodchem.2012.02.166.

Collins, S., York, DW, Kazmi, S., Mohammed, AK, 2019. Formation of wax walled microcapsules via double emulsion using cross membrane emulsification at elevated temperatures. J. Food Eng. 109739. doi:10.1016/j.jfoodeng.2019.109739

Cuenca, P., Ferrero, S., Albani, O., 2020. Preparation and characterization of cassava starch acetate with high substitution degree. Food Hydrocoll. 100, 105430. https://doi.org/10.1016/j.foodhyd.2019.105430

Estevez-Areco, S., Guz, L., Candal, R., Goyanes, S., 2020. Active bilayer films based on cassava starch incorporating ZnO nanorods and PVA electrospun mats containing rosemary extract. Food Hydrocoll. 108, 106054. https://doi.org/10.1016/j.foodhyd.2020.106054

Fernandes, RVB, Borges, SV, Botrel, DA, 2014. Gum arabic / starch / maltodextrin / inulin as wall materials on the microencapsulation of rosemary essential oil. Carbohyd. Polym. 101, 524-532. http://dx.doi. org/10.1016/j.carbpol.2013.09.083

Ferreira, TP, Haddi, K., Correa, RFT, Zapata, VLB, Piau, TB, Souza, LFN, Santos, SG, Oliveira, EE, Jumbo, LOV, Ribeiro, BM, Grisolia, CK, Fidelis, RR, Aguiar, RWS, 2019. Prolonged mosquitocidal activity of *Siparuna guianensis* essential oil encapsulated in chitosan nanoparticles. PLoS Negl. Trop. Dis. 9, 1-23. https://doi.org/10.1371/journal.pntd.0007624

Ferreira, TP, Oliveira, EE, Tschoeke, PH, Pinheiro, RG, Maia, AMS, Aguiar, RWS, 2017a. Potential use of Negramina (*Siparuna guianensis* Aubl.) essential oil to control wax months and its selectivity in relation to honey bees. Ind. Crops Prod*. 109*: 151-157. https://doi.org/10.1016/j.indcrop.2017.08.023

Ferreira, TP, Kuckelhaus, IGP, Tschoeke, PH, Cangussu, AS, Borges, JCM., Moura, WS, Aguiar, RWS, 2017b. Influence of seasonality on the yield and composition of the essential oil of *Siparuna guianensis* Aublet. Afr. J. Biotechnol. 16(29), 1611–618. https://doi:10.5897/ajb2017.16109

Finney, DJ, 1971. Probit analysys. 3th ed. Cambridge University Press, London. 25pp.

Fortunati, E., Gigli, M., Luzi, F., Lotti, N., Munari, A., Gazzano, M., Kenny, JM, 2016. Poly(Butylene cyclohexanedicarboxylate/diglycolate) random copolymers reinforced with SWCNTs for multifunctional conductive biopolymer composites. Express Polym. Lett. 10(2), 111–124. https://doi.org/10.3144/expresspolymlett.2016.12

Fuentes, C., Ruiz-Rico, M., Fuentes, A., Barat, JM, Ruiz, MJ, 2020. Comparative cytotoxic study of silica materials functionalised with essential oil components in HepG2 cells. Food Chem. Toxicol*.* 111858. https://doi.org/10.1016/j.fct.2020.111858

Glenn, GM, Klamczynski, AP, Woods, DF, Chiou,B., Orts, WJ, Imam, SH, 2010. Encapsulation of Plant Oils in Porous Starch Microspheres. J. Agricult. Food Chem. 58: 4180–4184. DOI:10.1021/jf9037826

González, A., Martínez, ML, Paredes, AJ, León, AE, Ribotta, PD, 2016. Study of the preparation process and variation of wall components in chia (*Salvia hispanica* L.) oil microencapsulation. Powder Technol*.* 301 (2016), 868-875. 10.1016/j.powtec.2016.07.02

Haddad, JG, Picard, M., Bénard, S., Desvignes, C., Desprès, P., Diotel, N., El Kalamouni, C., 2019. *Ayapana triplinervis* Essential oil and its main component Thymohydroquinone Dimethyl ether inhibit zika virus at doses devoid of toxicity in zebrafish. *Molecules*. 24(19), 3447. doi:10.3390/molecules24193447

Haddi, K., Turchen, LM, Viteri Jumbo, LO, Guedes, RN, Pereira, EJ, Aguiar, RWS, Oliveira, EE, 2020. Rethinking biorational insecticides for pest management: unintended effects and consequences. Pest Manag. Sci. 76: 2286-2293. doi:10.1002/ps.5837

Isman, BM, 2020. Botanical Insecticides in the Twenty-First Century—Fulfilling Their Promise?. Annu. Rev. Entomol. 65:233-249. https://doi.org/10.1146/annurev-ento-011019-025010

Kang, M., Cha, SH, Wijesinghe, WAJP, Kang, S, Lee, S., Kim, E., Song, CB, Jeon, Y., 2013. Protective effect of marine algae phlorotannins against AAPH-induced oxidative stress in zebrafish embryo. Food Chem. 138 (2–3), 950-955. https://doi.org/10.1016/j.foodchem.2012.11.005

Kishore, RS, Pappenberger, A., Dauphin, IB, Ross, A., Buergi, B., Staempfli, A., Mahler, HC, 2011. Degradation of polysorbates 20 and 80: studies on thermal autoxidation and hydrolysis. J. Pharmac. Sci. 100(2), 721-31. doi: 10.1002/jps.22290

Kumar, PM, Murugan, K., Madhiyazhagan, P., Kovendan, K., Amerasan, D., Chandramohan, B., Dinesh, D., Suresh, U., Nicoletti, M., Alsalhi, MSA, Devanesan, S., Wei, H., Kalimuthu, K., Hwang, J., Iacono, AL, Benelli, G., 2016. Biosynthesis, characterization, and acute toxicity of *Berberis tinctoria*-fabricated silver nanoparticles against the Asian tiger mosquito, *Aedes albopictus*, and the mosquito predators *Toxorhynchites splendens* and *Mesocyclops thermocyclopoides*. Parasitol. Res. 115(2), 751–759. doi:10.1007/s00436-015-4799-y

Liu, R., Sun, W., Zhang, Y., Huang, Z., Hu, H., Zhao, M., 2019. Preparation of starch dough using damaged cassava starch induced by mechanical activation to develop staple foods: Application in crackers. Food Chem. 271, 284-290. https://doi.org/10.1016/j.foodchem.2018.07.202

López, A., Castro, S., Andina, MJ, Ures, X., Munguía, B., Llabot, JM, Elder, H., Dellacassa, E., Palma, S., Domínguez, L., 2014. Insecticidal activity of microencapsulated *Schinus molle* essential oil. Ind. Crops Prod. 53, 209-216. https://doi.org/10.1016/j.indcrop.2013.12.038

Lourenço, AM, Haddi, K., Ribeiro, BM, Corrêia, RF, Tomé, HV, Santos-Amaya, O., Pereira, EJ, Guedes, RN, Santos, GR, Oliveira, EE, Aguiar, RWS, 2018. Essential oil of *Siparuna guianensis* as an alternative tool for improved lepidopteran control and resistance management practices. Sci. Rep. 8, 7215. https://doi: 10.1038/s41598-018-25721-0

Lucia, A., Girard, C., Fanucce, M., Coviella, C., Rubio, RG, Ortega, F., Guzmán, E., 2020. Development of an environmentally friendly larvicidal formulation based on essential oil compounds blend to control *Aedes aegypti* larvae: Correlations between physico-chemical properties and insecticidal activity. ACS Sus. Chem. Eng. 8, 29, 10995–11006. doi:10.1021/acssuschemeng.0c03778

Márquez-Gómez, M, Galicia-García, T., Márquez-Melendez, R., Ruíz-Gutierrez, M., Quintero-Ramos, A., 2017. Spray-dried microencapsulation of orange essential oil using modified rice starch as wall material. J. Food Process. Preserv. 42 (2), e13428. https://doi.org/10.1111/jfpp.13428

Mossa, ATH, Abdelfattah, NAH, Mohafrash, SMM, 2017. Nanoemulsion of camphor (*Eucalyptus globules*) essential oil, formulation, characterization and insecticidal activity against wheat weevil, *Sitophilus granaries*. *Asian J. of Crop Sci*., 9: 50-62. DOI: 10.3923/ajcs.2017.50.62

Moura, WS, Souza, SR, Campos, FS, Cangussu, ASR, Santos, EMS, Andrade, BS, Viana, KF, Haddi, K., Oliveira, EE, Nascimento, VL, Aguiar, RWS, 2020. Antibacterial activity of *Siparuna guianensis* essential oil mediated by impairment of membrane permeability and

replication of pathogenic bacteria. Ind. crops prod. 46, 1-9. https://doi.org/10.1016/j.indcrop.2020.112142

Moura, WS, Ascheri, DPR, 2018. Elaboration of biodegradable films of *Hedychium coronarium*. (1st ed.). Mauritius: New academic editions, 2018. v. 1. 125p.

Murúa-Pagola, B., Beristain-Guevara, CI, Martínez-Bustos, F., 2009. Preparation of starch derivatives using reactive extrusion and evaluation of modified starches as shell materials for encapsulation of flavoring agents by spray drying. J. Food Eng. 91(3), 380–386. https://doi.org/10.1016/j.jfoodeng.2008.09.035

NIST, Chemistry WebBook, 2018. NIST Standard Reference Database Number 69, Eds. P.J. Linstrom and W.G. Mallard, National Institute of Standards and Technology, Gaithersburg MD, 20899. https://doi.org/10.18434/T4D303

OECD (2013), Organization for Economic Co-operation and Development. *Test No. 236:* Fish Embryo Acute Toxicity *(FET) Test*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris. https://doi.org/10.1787/9789264203709-en

Oliveira, MS, Cruz, JN, Almeida da Costa, W., Silva, SG, Brito, MP, de Menezes, SAF, de Jesus Chaves Neto, AM, de Aguiar Andrade, EH, de Carvalho Junior, RN, 2020. Chemical Composition, Antimicrobial Properties of *Siparuna guianensis* Essential Oil and a Molecular Docking and Dynamics Molecular Study of its Major Chemical Constituent. Molecules. 25, 3852. https://doi.org/10.3390/molecules25173852

Pavela, R., Benelli, G., Canale, A., Maggi, F., Mártonfi, P., 2020. Exploring essential oils of Slovak medicinal plants for insecticidal activity: The case of Thymus alternans and Teucrium montanum subsp. Jailae. Food Chem. Toxicol. 138, 111203. https://doi.org/10.1016/j.fct.2020.111203

Pavela, R., 2015. Essential oils for the development of eco-friendly mosquito larvicides: A review. Ind. Crops Prod. 76, 174-187, https://doi.org/10.1016/j.indcrop.2015.06.050

Peres, MC, de Souza Costa, GC, dos Reis, LEL, da Silva, LD, Peixoto, MF, Alves, CCF, de Melo Cazal, C., 2020. In natura and nanoencapsulated essential oils from *Xylopia aromatica* reduce oviposition of *Bemisia tabaci* in *Phaseolus vulgaris*. J. Pest Sci. 93, 807-821. doi:10.1007/s10340-019-01186-6

Peterson, DL, Böröczky, K., Tumlinson, J., Cipollini, D., 2020. Ecological fitting: Chemical profiles of plant hosts provide insights on selection cues and preferences for a major buprestid pest. Phytochemistry. 176, 112397. doi:10.1016/j.phytochem.2020.112397

Portella, ACF, Murano, M., Ascêncio, SD, Siqueira, CA, Ferreira, TPS, Aguiar, RWS, 2014. Physical and chemical Characterization of essential oil of *Siparuna guianensis* Aublet. Quím. Nova. 37, 844-849. http://dx.doi.org/10.5935/0100-4042.20140136

Ribeiro, JS, Veloso, CM, 2021. Microencapsulation of natural dyes with biopolymers for application in food: A review. Food Hydrocoll. 112:106374. https://doi.org/10.1016/j.foodhyd.2020.106374

Rizzo, R., Lo Verde, G., Sinacori, M., Maggi, F., Cappellacci, L., Petrelli, R., Vittori, S., Morshedlood, M.R., Yvette Fofie, N.G.B., Benelli, G., 2020. Developing green insecticides to manage olive fruit flies? Ingestion toxicity of four essential oils in protein baits on *Bactrocera oleae*. Ind. Crops Prod. 143, 111884. https://doi.org/10.1016/j.indcrop.2019.111884

Samakradhamrongthai, R., Thakeow, P., Kopermsub, P., Utama-Ang, N., 2015. Encapsulation of *Michelia alba* D.C. Extract Using Spray Drying and Freeze Drying and Application on Thai Dessert from Rice Flour. Int. J. Food Eng. 1 (2). https://doi: 10.18178/ijfe.1.2.77-85

Sanjeewa, KKA, Kang, N., Ahn, G., Jee, Y., Kim, Y., Jeon, Y., 2018. Bioactive potentials of sulfated polysaccharides isolated from brown seaweed *Sargassum* spp in related to human health applications: A review. Food Hydrocoll. 81, 200-208. https://doi.org/10.1016/j.foodhyd.2018.02.040

Sun, JS, Feng, Y., Wang, Y., Li, J., Zou, K., 2020. α-Pinene, Caryophyllene and β-Myrcene from *Peucedanum terebinthaceum* Essential oil: Insecticidal and Repellent Effects on Three Stored-Product. Insects Rec. Nat. Prod. 14 (3), 177-189. DOI:10.25135/rnp.149.19.05.1287

Tappiban, P., Sraphet, S., Srisawad, N., Wu, P., Han, H., Smith, DR, Bao, J., Triwitayakorn, K., 2020. Effects of cassava variety and growth location on starch fine structure and physicochemical properties. Food Hydrocoll. 108, 106074. https://doi.org/10.1016/j.foodhyd.2020.106074

Tappiban, P., Smith, DR, Triwitayakorn, K., Bao, J., 2019. Recent understanding of starch biosynthesis in cassava for quality improvement: A review. Trends Food Sci. Technol. 83, 67-180. https://doi.org/10.1016/j.tifs.2018.11.019

Toledo, PFS, Ferreira, TP, Bastos, IMAS, Rezende, SM, Jumbo, LOV, Didonet, J., Andrade, BS, Melo, TS, Smagghe, G., Oliveira, EE, Aguiar, RWS, 2019. Essential oil from Negramina (*Siparuna guianensis*) plants controls aphids without impairing survival and predatory abilities of non-target ladybeetles. Environ. Pollut. 255:1, 113153. https://doi.org/10.1016/j.envpol.2019.113153

Tomazelli, O., Kuhn, F., Padilha, PJM, Vicente, LRM, Costa, SW, Boligon, AA, Scapinello, J., Nesi, CN, Dal Magro, J., Castellví, SL, 2018. Microencapsulation of essential thyme oil by spray drying and its antimicrobial evaluation against *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. Braz. J. Biol. 78, 2. https://dx.doi.org/10.1590/1519-6984.08716

Tongdeesoontorn, W., Mauer, LJ, Wongruong, S., Sriburi, P., Rachtanapun, P., 2011. Effect of carboxymethyl celulose concentration on physical properties of biodegradable cassava starch-based films. Chem. Central J. 5:6, 2011. https://doi.org/10.1186/1752-153X-5-6

Vicentini, N. M., 2003. Elaboration and characterization of edible films based on cassava starch for use in post-harvest. Thesis. UNESP, São Paulo, SP. 198p.

Zaitoon, A., Lim, L., Scott-Dupree, C., 2021. Activated release of ethyl formate vapor from its precursor encapsulated in ethyl Cellulose/Poly(Ethylene oxide) electrospun nonwovens intended for active packaging of fresh produce. Food Hydrocoll. 112: 106313. https://doi.org/10.1016/j.foodhyd.2020.106313

CHAPTER III - Safety and effects of a *Chiococca alba* root-based herbal medicine in patients with COVID-19: Phase 1 clinical trials

ABSTRACT

COVID-19 emerged at the end of 2019, evolving into a major pandemic and causing several deaths because there are no drugs capable of controlling the virus. The use of herbal medicines appears to be an alternative way to discover new molecules capable of containing the action of the Sars-CoV-2 virus. *Chioccoca alba* plants from the neotropical region have been widely studied for having molecules with known antiviral action. To understand the mechanism of action, the identified compounds from *C. alba* were modeled and tested in silico. In vitro studies were also carried out using Vero E6 cells to verify the antiviral action and cytotoxicity of the plant compounds. Clinical tests were then carried out to verify the safety and effects of compounds from the root of *C. alba.* Molecular docking results showed that vitexin and naringin molecules present -9.8 and -9.9 kcal/mol, respectively, when analyzed with the protein complex formed by ACE2 x Spike protein. Active site interactions were shown to be stable through molecular dynamics studies. In vitro studies on Vero E6 cells showed a selectivity index of 5000 for the methanolic extract of *C. alba*, a result far superior to other studies of this nature. Therefore, our clinical studies with humans are very promising, since at the end of the seventh day after the start of treatment, 64% of the patients who used the prepared tea had a negative result for the Sars-CoV-2 virus. They also showed a significant reduction in clinical symptoms, not showing symptoms such as fever, fatigue, myalgia and sore throat at the end of the treatment. Laboratory tests also showed that there was no significant difference for treatments with healthy patients, showing that there is safety in the use of products from the roots of *C. alba*. The results found in this study are very encouraging, demonstrating the safety of using the roots of *C. alba* in the treatment of COVID-19, as well as showing positive effects in the treatment, requiring further clinical trials to expand the number of participants.

1 INTRODUCTION

When the first case of coronavirus 2019 disease (COVID-19) was reported in December 2019 in Wuhan, China (Hu et al., 2020) it could not have been imagined that this disease would lead to a pandemic with a large number of deaths (Shi et al., 2021). Currently, the number of active confirmed cases continues to increase globally and their mortality rate can reach 16.4 per 100 confirmed cases in some countries (Zhou et al., 2021), and up to 78% in severe/critical cases (Zhou et al., 2020). To date, there is no specific therapy for COVID-19,

and some medical professionals have used off-label or compassionate therapies, such as lopinavir-ritonavir, chloroquine, convalescent plasma, and remdesivir (Holshue et al., 2020). No specific antiviral drug has been shown to be effective for the treatment of patients infected with the SARS-CoV-2 virus. Some known antiviral drugs, such as Remdesivir, have undergone clinical trials in patients with moderate to severe COVID-19 infections, however, the results have not been conclusive, suggesting that Remdesivir is not the best drug for COVID-19 (Beigel et al., 2020; Wang et al., 2020).

Some treatments based on herbal medicines have not been studied as an alternative for identifying compounds that may act on the mechanisms of action of the Sars-CoV-2 virus (Mhatre et al., 2021). Researchers in China have been conducting several studies using traditional Chinese medicine plants with promising results (Shi et al., 2021; Yan et al., 2021). Additionally, researchers from India have shown positive results for the use of plant compounds in the treatment of Covid-19 (Devpura et al., 2021). The neotropical region has vast biodiversity, and advances in studies have shown some plants with promising results in the treatment of some viruses (Amparo et al., 2021).

Chiococca alba (L.) Hitchc. (Rubiaceae), popularly known as caninana, is a plant originating from the neotropical region, and its roots are used in traditional medicine with good antiviral, anti-rheumatic, anti-inflammatory and antibacterial activity (Mors et al., 2000). The chemical compounds present in their structures also demonstrate pharmacological activities mainly due to their composition based on flavonoids, alkaloids and saponins (Borges et al., 2009). Toxicological studies have shown the absence of mutagenic activity in the ethanol extract of *C. alba* roots and low acute and subacute toxicity by the oral route (Gazda et al., 2006).

In this sense, the present work aimed to characterize the main compounds present in the roots of *C. alba*. *In silico* analysis was carried out to identify the mechanisms of action of the compounds in the plant on the different targets existing in the Sars-CoV-2 virus. Develop *in vitro* assays to verify the antiviral action and cytotoxicity in human cells. The realization of phase 1 clinical trials in humans to identify the effects and safety of *C. alba* root against COVID-19.

2 MATERIALS AND METHODS

2.1 Extraction and characterization of *C. alba* extract

Roots of *C. alba* collected in Formoso do Araguaia (11° 47'48 "latitude S, 49° 31'44" longitude W), Tocantins, Brazil, were used. The present investigation was registered in SISGEN under the number A6CD4E6. Plant *C. alba* was identified by the Porto Nacional Herbarium of the Federal University of Tocantins with the number HTO-11,160.

To obtain extracts of *C. alba* root, powder was used at a ratio of 1 g: 10 mL of MeOH (methanol) in a 1 L amber flask. The root powder was in contact with the solvent for 48 h in a shaker with constant stirring at 120 rpm. The crude extract was filtered and subsequently the volume of solvent used was reduced with the aid of a rotary evaporator. The samples were subjected to a pressure of 600 mmHg, rotation of 80 rpm and a heating bath at a temperature of 45°C (Negri et al., 2012).

The chemical profile of the *C. alba* extract was analyzed by HPLC, using a chromatograph (Shimadzu®, Kyoto, Japan) equipped with an LC – 10AT pump to pump the mobile phase, DGU – 14A degasser, SPD – 10A UV-vis detector, column oven CTO – 10A, manual injector (Rheodyne) with 20 µl loop and a CLASS SLC integrator – 10A. The extract and standard solutions were prepared with methanol and filtered through Millipore® membrane (pore size 0.22 µm). Separation was performed using a gradient system, using a C18 Phenomenex Luna 5 µm (250 × 4.6 mm2) column, reversed phase, with direct-connected C18 Phenomenex Security Guard cartridges (4 × 3.0 mm2), at 22°C. Detector response was recorded and integrated using Class-VP software. Mobile phase A consisted of 0.1% phosphoric acid in Milli-Q water and mobile phase B consisted of 0.1% phosphoric acid in Milli-Q water / acetonitrile / methanol in the ratio 54:35:11 (v /v). The program gradient was: 0 to 5 min, 0% B; 5 to 10 min, 30% B; 10 to 20 min, 40% B; 20 to 60 min 40% B; 60 to 70 min, 50% B; 70 to 90 min 60% B; 90 to 120 min 100% B. Flow: 1 mL / min, temperature: 22 °C. UV detection was performed at 280 nm (Soares et al., 2017).

2.2 *In silico* interaction analysis

2.2.1 Molecular docking

An *in silico* study of interactions between C. alba molecules and human SARS-Cov-2 and ACE2 receptors was carried out. The X-ray structures of the 3CL-protease, Mpro protease, RpRd and ACE2 x Spike protein proteases were obtained from the Protein Data Bank (https://www.rcsb.org/) (PDB ID: 6LU7, 6Y2E, 6NUR and 6M0J, respectively). *C. alba* molecules were modeled using Marvin Sketch 18.10, ChemAxon ((http://www.chemaxon.com) and, receptors and ligands prepared for the molecular coupling process using Autodock Tools 1.5.7 (Sanner, 1999 Nine coupling positions were generated for each ligand interacting with the target protein, returning affinity energy values (kcal/mol) using AutoDock Vina in coupling calculations (Trott & Olson, 2010). were analyzed using PyMOL 2.0 (Schrodinger, 2018) and Discovery Studio 4.5 ((Dassault Systemes BIOVIA, 2017) to select the best position for each ligand within the protein target (Moura et al., 2020).

2.2.2 Molecular dynamics

Molecular dynamics simulations were performed using the MDWeb server (Hospital, et al. 2012), and the molecular docking PDB files were used to prepare the simulation base. The structure was prepared using Gromacs full MD Setup with the AMBER-99SB * force field, as it satisfactorily describes the molecular behavior of proteins. The molecular dynamics simulation process was carried out according to the set of steps that included cleaning the structure, fixing the side chains of the complex, adding the solvent box, minimal energy and balancing the system to receive the minimized structure as an outlet (Chaudhary et al., 2019). These simulations were performed in constant volume (NVT) (Oliveira et al., 2019; Oliveira et al., 2020). In the equilibrium stage, the systems were subjected to a simulation of 2.5 ps with a temperature of 300 K and constant pressure. After the generation of the protein-ligand complex, the water molecules and ions were removed to reduce the size of the system and the dry trajectory was recovered to trace the mean quadratic deviation (RMSD).

2.3 Cytotoxic and antiviral activity assays

The *C. alba* methanol extract was dissolved at the indicated concentrations and serially diluted in DMSO. Immediately before plating, the compounds were diluted 33,33 x in PBS, and 10 µl of these solutions was transferred to assay plates.

Vero E6 cells were plated onto 384-well plates in high DMEM, and incubated for 24 h. After incubation (37ºC, 5% CO2), the compounds were added to the plate. The first concentration tested was 10 micrograms/mL and the subsequent concentrations consisted of a serial dilution of the first by a dilution factor equal 2. After 33h of incubation (37ºC, 5% CO2), plates were fixed with 4% PFA and subjected to indirect immunofluorescence. After washing twice with PBS pH 7.4, plates were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS (BSA-PBS) for 30 min at RT and washed twice with PBS. As a primary antibody, serum from a convalescent COVID-19 Brazilian patient diluted 1:1000 in PBS was used to detect Sars-CoV-2 infection in Vero cells. The primary antibodies were incubated for 30 min, and plates were washed twice with PBS. As secondary antibodies, goat anti-human IgG labeled with Alexa 488 (Thermo Scientific) was diluted 1:2000 in PBS and incubated for 30 min with 5 µg/mL 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) in PBS to stain nuclei. The plates were washed twice with PBS and imaged in the Operetta High Content Imaging System (Perkin Elmer) using a 20x magnification objective. Seven images were acquired per well.

2.3.1 Data analysis

Acquired images were analyzed in Harmony software (Perkin Elmer), version 3.5.2. Image analysis consisted of identifying and counting Vero E6 cells based on nuclear segmentation and viral infection based on the cytoplasmic staining detected by the immunofluorescence assay. The infection ratio (IR) was calculated as the ratio between the number of infected cells and the number of total cells counted in each well. The cell survival rate was calculated as the number of cells counted in each well divided by the average number of cells in the positive control (DMSO-treated infected cells) wells, multiplied by 100. The antiviral activity was determined by the normalization of the IR to the negative control (DMSO-treated infected and noninfected cells), as described. Concentration-response curves were plotted using the normalized activity and cell survival of each concentration. These two parameters were used to calculate the concentration of EC_{50} and CC_{50}, compound concentrations that reduced the infection ratio, and cell survival in 50%, respectively, compared to nontreated infected controls of each compound using GraphPad Prism version 7.0 (GraphPad Software, USA).

2.4 Clinical trials

2.4.1 Test design and supervision

This phase 1 clinical study was registered with the research ethics committee under CAAE number 43870721.6.0000.5519 and approved according to opinion number 4.602.901. This study was also registered in the Brazilian Registry of Clinical Trials (ReBEC) under the number RBR-10b8bzpd.The study was conducted in accordance with the principles of the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference on Harmonization.

The study was carried out in a single center, the Municipal Triage Center of Gurupi-TO - CMTG. Patients with symptoms of COVID-19 who presented at the CMTG initially underwent a medical evaluation, which indicated the need for a Swab RT-PCR type test to identify the Sars-CoV-2 virus infection. This patient was then invited to participate in the research by signing the Informed Consent Form (TCLE).

2.4.2 Patient selection

Patients over 18 years of age were selected, with the exclusion criteria being pregnancy or lactation, known hypersensitivity to the extract to be administered, active cancer, carriers of the human immunodeficiency virus, patients undergoing solid organ or bone marrow transplantation or in use of immunosuppressive drugs, bacterial infection at randomization,

sepsis or septic shock related to bacterial infection at randomization, inability to use medication by mouth or nasoenteral tube, known liver failure or advanced heart failure (New York Heart Association [NYHA] class) III or IV). (Cao et al., 2020).

2.4.3 Sample preparation

Phytotherapics were produced from collected *C. alba* roots, which were crushed in a willye Star FT 50 knife crusher (Fortinox, Piracicaba, Brazil) to form a root powder.

Sachet treatment was prepared using 2g of *C. alba* root powder in a sachet made of TNT material with dimensions of 8x7 cm. Sachets were placed in identified boxes and sealed with plastic material. Patients were instructed to use the sachet by heating the water to a boil, placing 200 mL of water in a container and then infusing the sachet for 6 min.

Tea treatment was prepared in accordance with the provisions of the Brazilian Pharmacopoeia (Brazil, 2019), using the proportion of 2g powder of *C. alba* root per 200 mL of water. The water was heated to a temperature of 95°C, and the powder was added directly to the water and kept in contact for 6 min. After this period, the solution was filtered through TNT material, and the prepared tea was placed in a plastic container and stored in a refrigerator at a temperature of 4°C. The patient was instructed to use 200 mL of the prepared tea.

Placebo treatment was prepared using inert material in powder and liquid form, with similar flavor and color to the other treatments, using artificial colorant (mix duas rodas, São Bernardo do Campo, SP).

2.4.4 Randomization

Forty-eight patients were selected to participate in the tests. Selected patients were randomly assigned in a 1:1:1 ratio to receive treatment with sachet, prepared tea and placebo using randomization and double-blind methodology through sample coding. The patient used the selected treatment for a period of 5 days, being used twice a day, giving a total of 10 doses.

Healthy patients with negative results for COVID-19 were also selected for treatment with herbal medicine to evaluate the safety of the product produced with the roots of *C. alba*.

2.4.5 Outcome Measures

Nasopharyngeal swab samples were collected by professionals from the Municipal Health Department, according to the local protocol, and these samples were identified and sent to the Central Public Health Laboratory of Tocantins - LACEN. LACEN performed the quantitative RT-PCR test using the Allplex^{TM} 2019-nCoV Assay Kit (seagene) according to the

parameters established by the manufacturer. QuantStudio 5 amplification equipment (Thermo Fisher Scientific, Waltham, USA) and KingfisherTM Flex extraction equipment (Thermo Fisher Scientific, Waltham, USA) were used.

Primary outcome measures were performed through the RT-PCR test, which was performed on the 1st day, before the beginning of the use of the samples and repeated on the 7th day, after completing the cycle of using the samples (Zhou et al., 2020). In addition, the results of clinical symptoms were reported by patients on a daily basis. Patients were monitored daily through a telephone call where they reported the main symptoms and were evaluated during the 5 days of treatment.

Secondary outcome measures were carried out through the collection of blood from patients and tests performed in the laboratory "Clinical Laboratory Analysis". The laboratory tests performed were: glutamic-oxaloacetic transaminase – GOT (kinetic method) (Reitman & Frankel, 1957), glutamic-pyruvic transaminase - GPT (kinetic method) (Reitman & Frankel, 1957), creatinine (Jaffe method) (Jaffe, 1886), urea (enzymatic method) (Bergmeyer, 1985) and C-reactive protein (latex agglutination method) (Singer et al., 1957), with modifications according to the protocols by the laboratory. The exams were performed on the 1st day, before starting to use the samples, and repeated on the 7th day, after completing the cycle of using the samples.

2.5 Statistical analysis

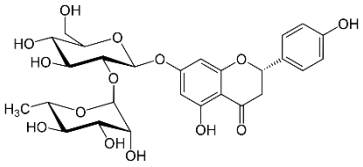
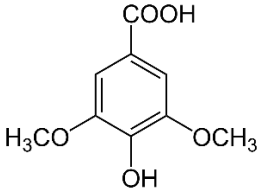
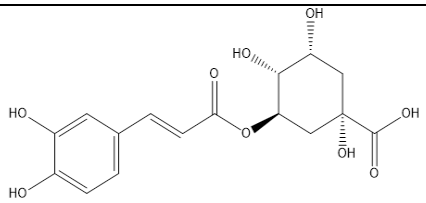
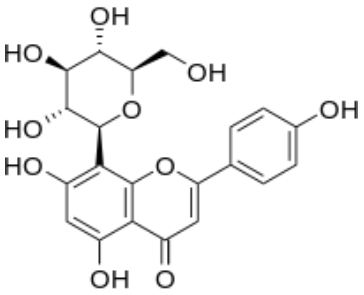
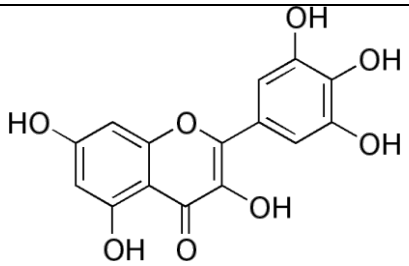
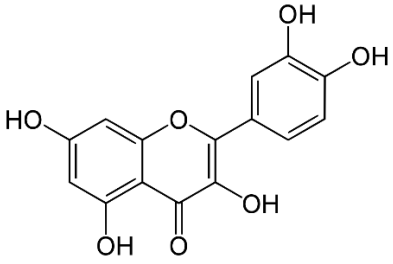
Continuous variables are described as the mean ± standard deviation. The χ^2 test was used to analyze categorical data, while comparisons between laboratory tests were made between baselines and group results using a t-test with Assistat 7.7 software (Silva & Azevedo, 2016). For all comparisons, differences were tested with two-tailed tests, and $p < 0.05$ was considered statistically significant.

3 RESULTS

3.1 Chemical composition of *C. alba* methanol extract

Chemical analysis using HPLC identified 6 (six) main components in the methanol extract of *C. alba* (Table 1).

Table 1. Chemical profile of *C. alba* methanol extract identified by HPLC

Order	Compound	Chemical structure	Molecular formula	Molar mass (g/mol)
1	Naringin		$C_{27}H_{32}O_{14}$	580.54
2	Syringic acid		$C_9H_{10}O_5$	198.17
3	Chlorogenic acid		$C_{16}H_{18}O_9$	354.31
4	Vitexin		$C_{21}H_{20}O_{10}$	432.38
5	Myricetin		$C_{15}H_{10}O_8$	318.23
6	Quercetin		$C_{15}H_{10}O_7$	302.23

3.2 *In silico* interaction of *C. alba* methanol extract and human and Sars-CoV-2 receptors

The molecular docking results presented in table 2 show that the molecules vitexin and naringin had better affinity energy with the proposed targets. Vitexin had an affinity energy value of -9.8 kcal/mol for the ACE2 x Spike receptor and naringin a value of -9.9 kcal/mol.

Table 2 - Affinity energy between the main ligands of the botanical sources of this project and Sars-CoV-2 and human receptor receptors

	RdRp	3CL-Protease	M^{pro} Protease	ACE2 x Spike
Ligand	kcal/mol			
Vitexin	-8.5	-7.2	-7.7	-9.8
Naringin	-8.8	-7.0	-8.3	-9.9
Chlorogenic acid	-6.6	-6.0	-6.1	-6.5
Myricetin	-7.2	-6.9	-8.0	-7.4
Myricetin	-6.9	-6.0	-7.2	-7.0
Syringic Acid	-4.1	-4.0	-4.2	-4.6

The naringin ligand showed interactions with the van der Waals human ACE2 protein (PHE40, HIS345, ALA348, ARG393, ASN394), conventional hydrogen bond (SER44, SER47, ASP350, ASP382, TYR385, HIS401), carbon hydrogen bond (HIS378), pi-alkyl (TRP349) and pi-sigma (THR347). The ligand vitexin showed interactions with human van deer Waals ACE2 (ILE418, ASP405), hydrogen conventional bond (ARG403, GLN409, LYS417, GLY496), carbon hydrogen bond (GLU406, TYR495), pi-alkyl (TYR505), also showed interactions with the van der Waals type Sars-CoV-2 Spike protein (LYS353), conventional hydrogen bond (HIS34) and pi-cation (HIS34).

Figure 1 shows the complex formed between the ACE2 x Spike proteins and the ligands vitexin and naringin.

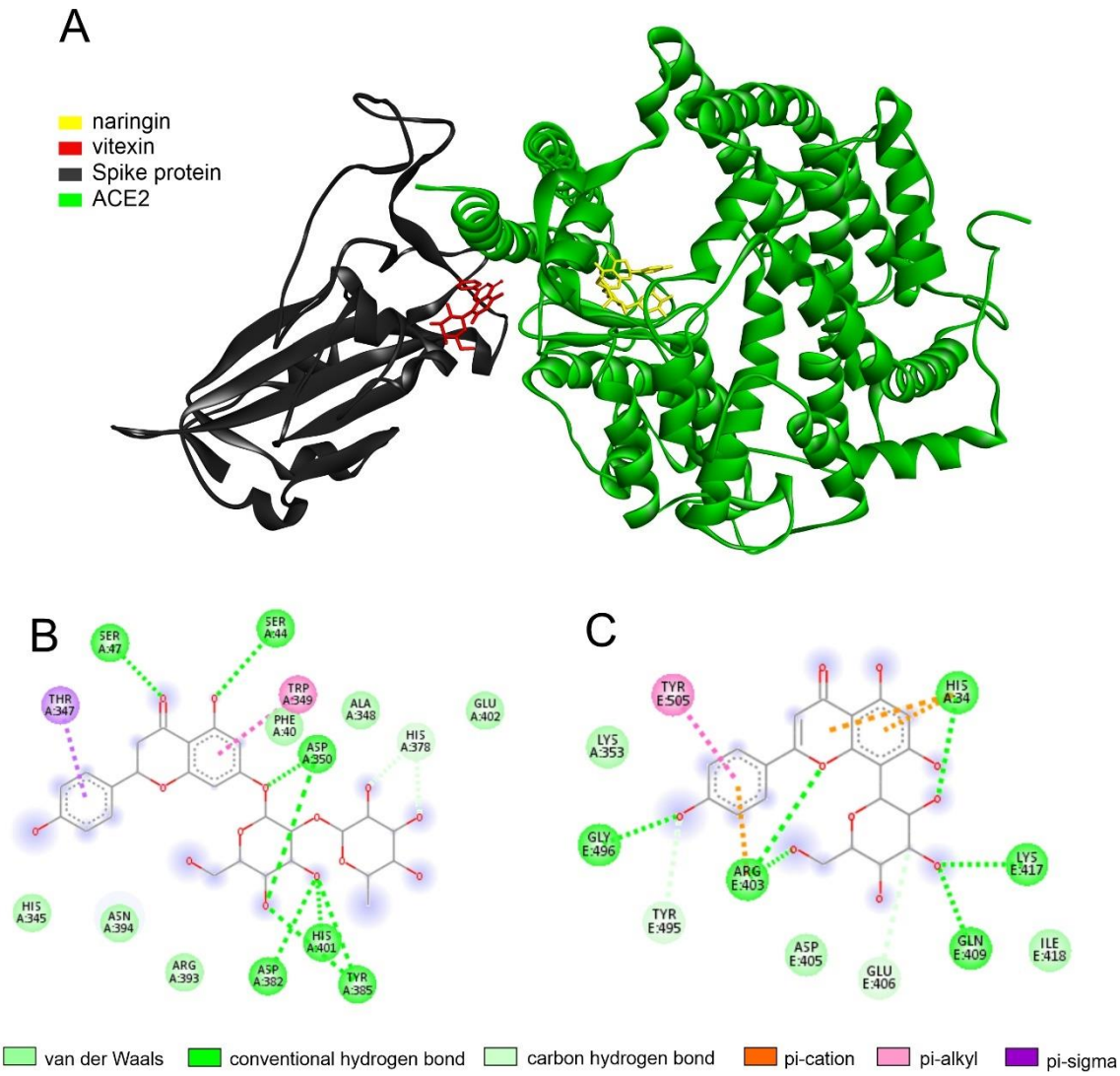


Figure 1 – Complex formed by (a) ACE2 x Spike protein receptors with the ligands vitexin and naringin and 2d interaction map of the ligands (b) naringin and (c) vitexin.

3.3 Molecular Dynamics Results

Molecular dynamics analysis of the complex formed by the ACE2 x Spike protein and the naringin and vitexin ligands, which presented better affinity energy in the study of molecular docking, was performed to identify the stability of the interactions formed.

Spatial RMSD calculated for the average position of each amino acid residue of the formed complex confirmed structural stabilization (Figure 2). The highest RMSD values were below 1.0 Å, indicating that the residues that underwent the greatest changes were those only in the regions corresponding to the loops. Furthermore, residues in the region of active sites exhibited even lower RMSD values (HIS34, LYS417), revealing greater stability in these areas.

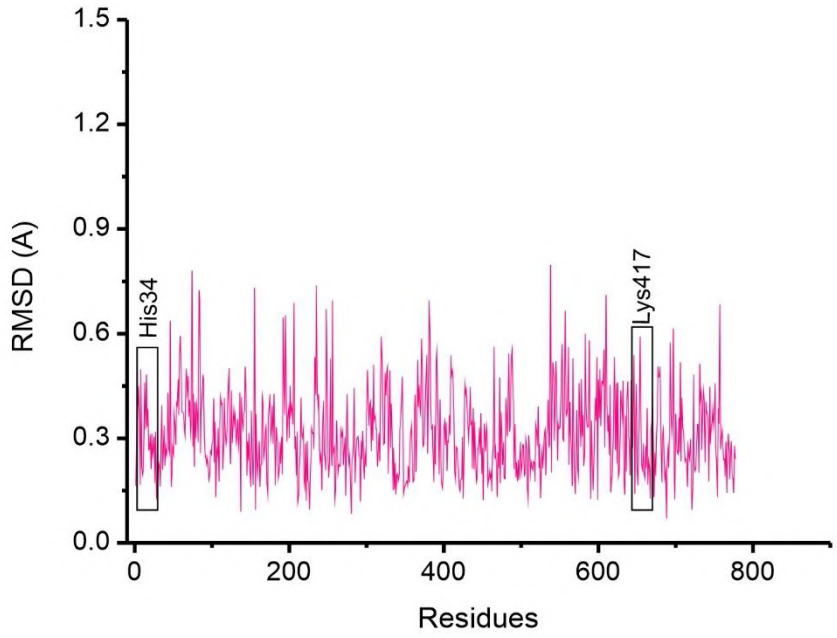


Figure 2 - Molecular dynamic simulation plots representing the mean quadratic deviation (RMSD) structural deviation per residue of the complex formed by ligands naringin, vitexin and the ACE2 x Spike protein.

3.4 Evaluation of *C. alba* extract with anti-Sars-CoV-2 activity

The results of the Sars-CoV-2 inhibition tests demonstrate that the methanol extract of *C. alba* has good anti-Sars-CoV-2 activity at low concentrations and still low cytotoxicity to Vero E6 cells (Figure 2). Table 3 shows the EC_{50} and CC_{50} results for the methanol extract *C. alba* and Chloroquine (control). The extract of C. alba showed a high level of selectivity against Sars-CoV-2 with a value equal to 5000.

Table 3 – EC_{50} values between the methanol extract of *C. alba* and Sars-Cov-2, CC_{50} between the methanol extract of *C. alba* and Vero E6 cells and selectivity index (SI).

	EC_{50} (mg/mL)	CC_{50} (mg/mL)	SI
C. alba extract	0.002	> 10.00	5000
Chloroquine	0.03	>20.00	667

EC_{50} = concentração da amostra que causa uma redução de 50% na infecção do vírus, em comparação com controles infectados;
CC_{50} = concentração da amostra que causa uma redução de 50% no número de células hospedeiras, em comparação com controles infectados.
SI: Selectivity index, determined by the ratio of CC_{50}/EC_{50}. This indicates how specific the antiviral activity is.

The dose-response curves for anti-Sars-Cov-2 activity and cell survival for the methanol extract of *C. alba* are shown in Figure 3.

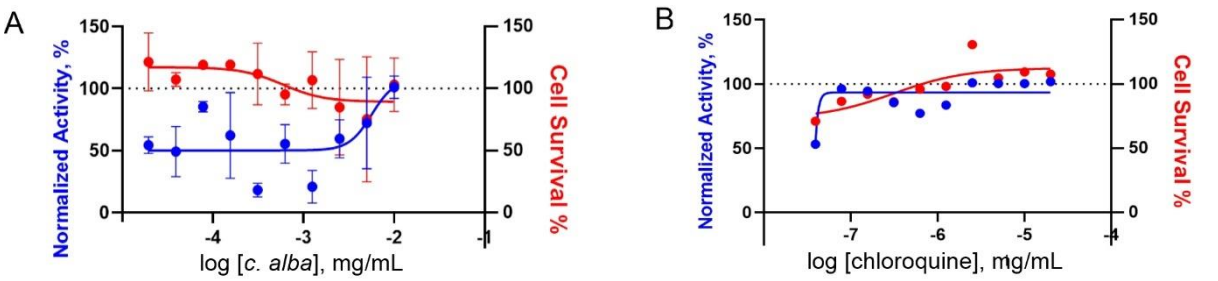


Figure 3 – Cell survival and anti-Sars-CoV-2 activity results of (a) methanol extract of *C. alba* and (b) chloroquine.

3.5 Results of clinical trials with *C. alba* roots products

A total of 48 patients participated in the clinical trials, distributed as described in table 3:

Table 3 - Profile of patients participating in clinical trials

Population	Sachet	Tea	Placebo	Unaffected
Number of patients (Male/Female)	14 (10/4)	11 (6/5)	11 (7/4)	12 (9/3)
Age	37.4 (±11.3)	38.7 (±10.9)	29.4 (±9.9)	33.5 (±8.7)

* Mean ± Standard deviation.

Patients who participated in the clinical trials underwent the RT-PCR test before starting treatment to confirm infection by the Sars-CoV-2 virus, and after treatment, on the seventh day, 64% of patients who used the tea prepared showed a negative RT-PCR test. The number of patients who used the sachet that tested negative was 50% and for the placebo group it was 36% at the end of the seventh day (Figure 4).

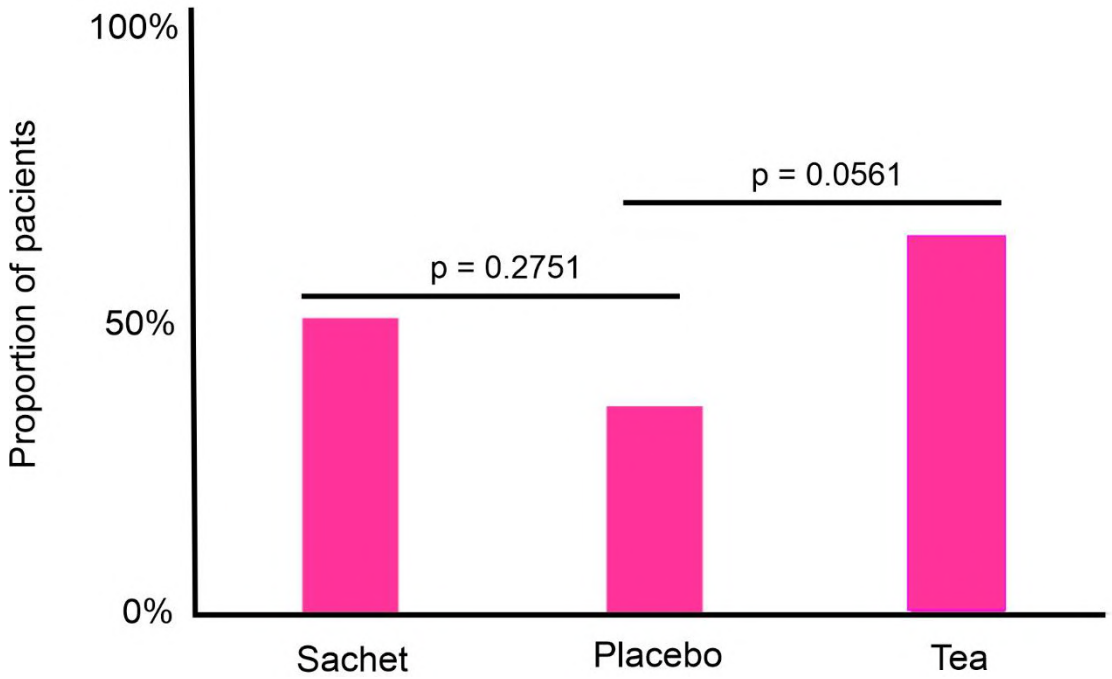


Figure 4 – Proportion of patients with negative RT-PCR results after the seventh day of treatment initiation. The p-value for the χ^2 test at the 5% level is represented.

Table 4 shows the values of the cycle threshold (CT) of the RT-PCR exams performed before the start of treatment and after the seventh day of treatment.

Table 4 – CT value for RT-PCR exams before start and after treatment.

	CT start	CT after treatment
Sachet	23.9 (±5.9)	29.9 (±4.3)
Tea	24.3 (±2.8)	30.7 (±4.2)
Placebo	23.9 (±5.3)	27.3 (±2.7)

* Mean ± standard deviation

After starting treatment, patients were monitored daily to check the evolution of clinical symptoms during the five days of treatment. Every day, they reported the clinical situation concerning the symptoms by telephone: cough, asthenia, fever, fatigue, anosmia, ageusia, myalgia, headache and sore throat, as shown in figure 5. At the end of the treatment, none of the patients who made use of the sachet reported symptoms of fever, fatigue, myalgia and sore throat. Of those who used tea prepared at the end of the treatment, none reported fatigue, myalgia or sore throat. While patients who used placebo treatment reported having some of the symptoms described.

Two patients who participated in the research and used the placebo treatment, after the seventh day of treatment had to be admitted to the health unit to receive supplemental oxygen, but approximately nine days after admission, they were discharged from the hospital.

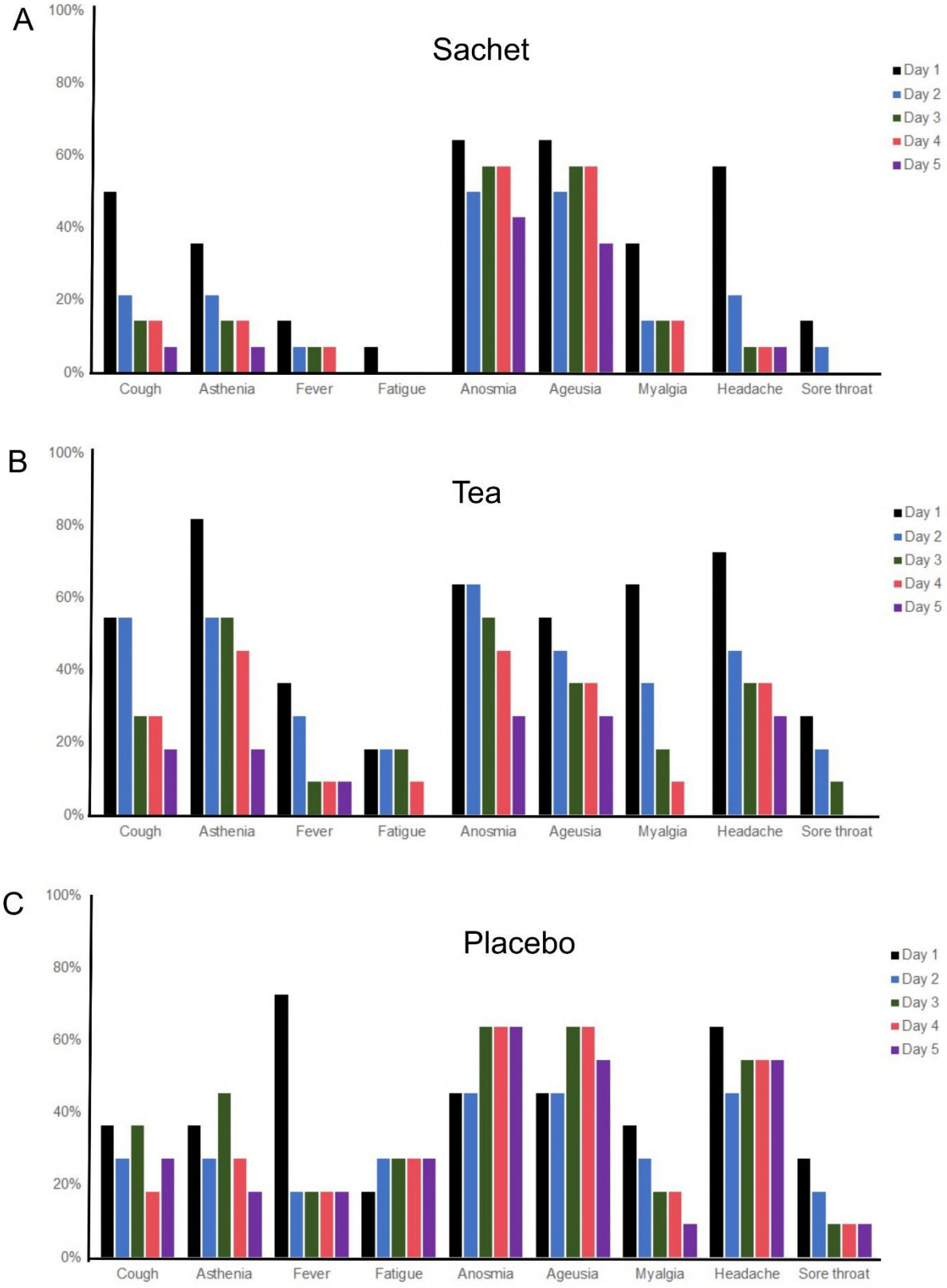


Figure 5 – Clinical results reported by patients during five days of treatment with the different study samples.

Laboratory tests were also performed before treatment to serve as a baseline and comparison of the safety of the samples. After the seventh day the laboratory tests were repeated as described in figure 6. The results for the unaffected patients demonstrate that the treatments with sachet and tea prepared using *C. alba* root are safe to use, since there were no significant variations of the test results. There was a significant difference between treatments only for creatinine ($p < 0.001$).

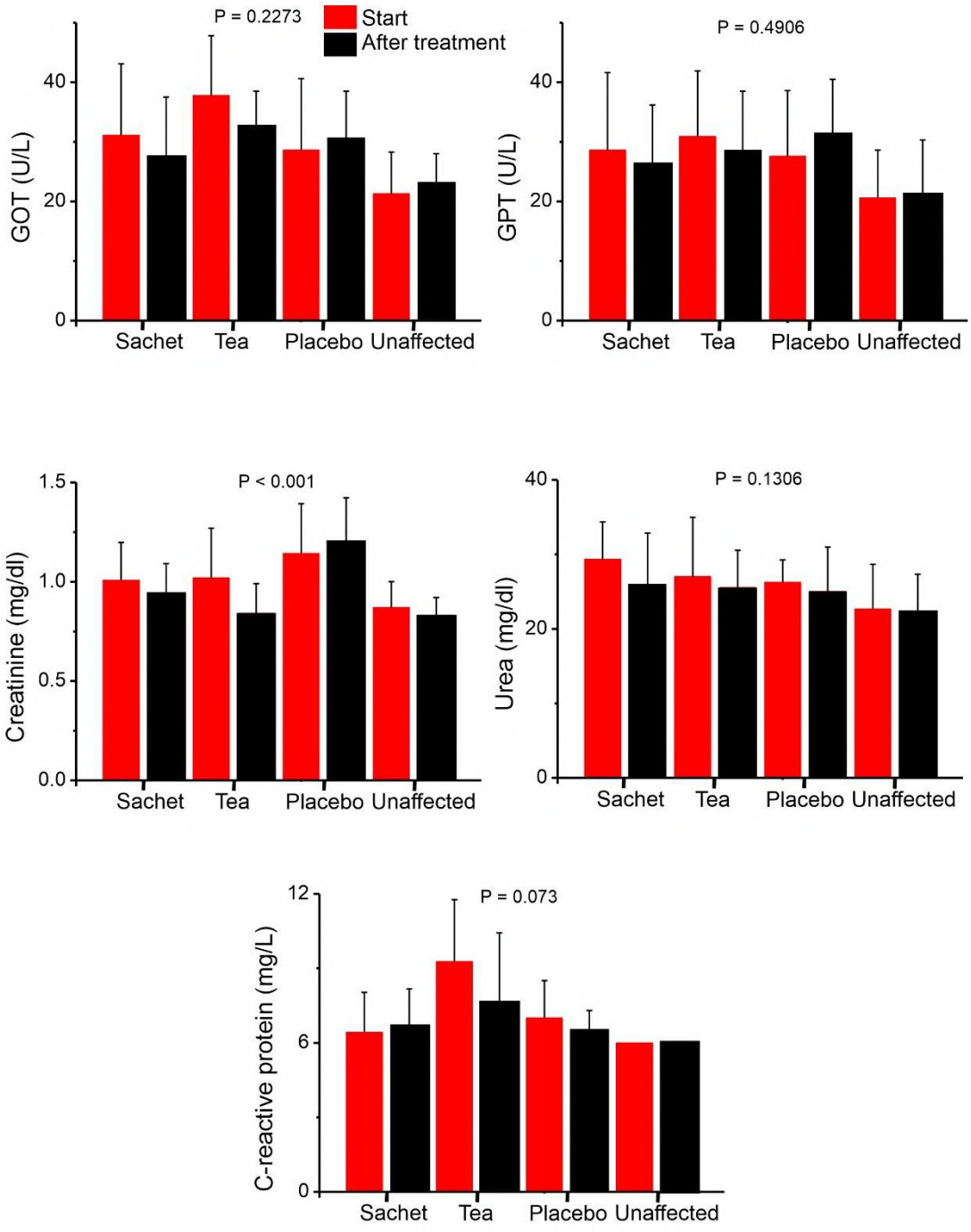


Figure 6 – Laboratory tests performed before the start of treatment and after the seventh day of treatment. The standard deviation is represented on the bar for each sample and p-value for the t test at the 5% level. Reference values for the GOT (ref=40), GPT (ref=40), creatinine (ref= 1.4), rrea (ref=50) and C-reactive protein (ref<6).

4 DISCUSSION

Our results showed the importance of searching for new herbal medicines in an attempt to find a solution for COVID-19, since there is great biodiversity in the neotropical region. The *C. alba* plant presented molecules that have antiviral characteristics in its composition. In silico studies show us the possible mechanism of action of these molecules against the complex formed between the ACE2 x Spike protein, where the region of the active site presents stability in interactions. In vitro tests also demonstrated the good selectivity of the methanol extract of *C. alba* when tested against Sars-CoV-2 infection in Vero E6 cells. Furthermore, clinical trials in humans demonstrated safety in use since laboratory results did not show significant differences and patients with negative results after the seventh day were higher for patients who used treatments with *C. alba* roots.

The methanol extract of *C. alba* contains the vitexin molecule, which is recognized to have pharmacological properties, including anticancer, anti-inflammatory and antiviral properties (He et al., 2016; Sancesario & Bernardini, 2018; Krishnan & Kang, 2019). This molecule is commonly found in medicinal plants such as pearl millet, hawthorn, bamboo, and *Ficus deltoidea* (Yahaya et al., 2020). *C. alba* also has a naringin molecule that has antiviral activity against dengue virus (DENV-2) (Zandi et al., 2011) and HSV-1 and HSV-2 (Lyu et al., 2005). This molecule is found in citrus fruits such as oranges and passion fruit (Sehrawat et al., 2021).

These molecules are the target of in silico studies to identify mechanisms of action against Sars-CoV-2, showing good results for interactions carried out through molecular docking. Our results demonstrated affinity energy values for the vitexin molecule of -9.8 kcal/mol and -9.9 kcal/mol for naringin, while Sachdeva et al. (2020) found a values of -6.76 and -4.02 kcal/mol for the drugs remdesevir and chloroquine, respectively, when tested for the ACE2 x Spike Protein target. This result shows that the components of the *C. alba* extract show promising results due to their good affinity energy and their interactions with the amino acids in the active site of human ACE2 proteins and the Spike protein of Sars-CoV-2 (Lan et al., 2020).

We observed that the methanol extract of *C. alba* presented a selectivity index (SI) result with a value of 5000. The SI is the value of the ratio between CC_{50} and EC_{50}, and high value for this parameter indicates that the compound has a better action against the virus in relation to human cells, indicating that it is an efficient and safe compound (Alnajjar et al., 2020). Although controversial, chloroquine is used as an anti-Sars-CoV-2 agent because it has good in vitro results, serving as a control for comparison of experiments (Wang et al., 2020) Our experiment presented a value for the selectivity index of 667 for chloroquine, a value well below that found for the methanol extract of *C. alba*.

Our clinical study was performed as an adjunctive treatment to identify the safety and possible effects of samples from the root of *C. alba*. We observed that 64% of the patients who used the prepared tea tested negative for COVID-19 on the seventh day, while 50% of the patients who used the sachet tested negative and 36% of the placebo patients tested negative. This suggests that the way the compound is administered will influence the results, since the tea was prepared under the right conditions, following a standardization in its production. It is also worth noting that the patients who used the products from the roots of *C. alba* (Sachet and Tea) did not progress to hospitalization.

Patients who used traditional herbal medicines known as Ayurvedic had reduced COVID-19 symptoms in 83.33% of patients after 13 days of use (Balkrishna et al., 2021). Patients who used the sachet and prepared tea treatment did not present symptoms of fever, fatigue, myalgia and sore throat in a shorter period (5 days), indicating a significant improvement with the treatments.

Laboratory tests indicated a slight decrease in the levels of GOT, GPT, creatinine and urea, both for the treatment with sachet and for the treatment with the prepared tea, but the average of the results is within the expected normal standards. A higher value of these indices at the start of treatments may be related to liver damage caused by COVID-19 (Senegaglia et al., 2021). Patients who tested negative for COVID-19 and used the samples did not show significant differences in the results of laboratory tests, thus demonstrating the safety of treatments using compounds from the root of *C. alba*.

5 CONCLUSION

Compounds from *C. alba* showed a good selectivity index *in vitro* tests for Sars-CoV-2 infection in Vero E6 cells. Phase 1 clinical trials showed good results, as *C. alba* root products tended to reduce the clinical symptoms of COVID-19, as well as a better reduction in positive results in the RT-PCR test. Laboratory tests with uninfected patients showed that *C. alba* does not present adverse results and does not present possible liver damage, with promising results for new phases of studies.

REFERENCES

Alnajjar, R.; Mostafa, A.; Kandeil, A.; Al-Karmalawy, A.A.(2020). Molecular docking, molecular dynamics, and in vitro studies reveal the potential of angiotensin II receptor blockers to inhibit the COVID-19 main protease. Heliyon. 6, 12, e05641. https://doi.org/10.1016/j.heliyon.2020.e05641

Amparo, T.R.; Seibert, J.B.; Silveira, B.M. et al. (2021) Brazilian essential oils as a source for the discovery of a new anti-COVID-19 drug: a review guided by an in silico study. Phytochem Rev . https://doi.org/10.1007/s11101-021-09754-4

Balkrishna,A.; Bhatt,A.B.; Singh, P.; Haldar, S.; Varshney, A. (2021) Comparative retrospective open-label study of ayurvedic medicines and their combination with allopathic drugs on asymptomatic and mildly-symptomatic COVID-19 patients. Journal of Herbal Medicine. 29,100472. https://doi.org/10.1016/j.hermed.2021.100472

Beigel, J.H.; Tomashek, K.M.; Dodd, L.E.; Mehta, A.K.; Zingman, B.S.; Kalil, A.C.; Hohmann, E.; Chu, H.Y.; Luetkemeyer, A.; Kline, S.; Lopez de Castilla, D.; Finberg, R.W.; Dierberg, K.; Tapson, V.; Hsieh, L.; Patterson, T.F.; Paredes, R.; Sweeney, D.A.; Short, W.R.; Touloumi, G.; Lye, D.C.; Ohmagari, N.; Oh, M.; Ruiz-Palacios, G.M.; Benfield, T.; Fätkenheuer, G.; Kortepeter, M.G.; Atmar, R.L.; Creech, C.B.; Lundgren, J.; Babiker, A.G.; Pett, S.; Neaton, J.D.; Burgess, T.H.; Bonnett, T.; Green, M.; Makowski, M.; Osinusi, A.; Nayak, S.; Lane, H.C. (2020) Remdesivir for the treatment of covid-19 — preliminary report N. Engl. J. Med (2020), pp. 1-12. Doi: 10.1056/nejmoa2007764

Bergmeyer, H.U. (1985) Methods of Enzymatic Analisis. 9, 449-453, VCH Publishers, Florida-US.

Borges, R. M. et al. (2009). Two new oleanane saponins from Chiococca alba (L.) Hitch.. Journal of the Brazilian Chemical Society 20, (9), 1738-1741. ttps://doi.org/10.1590/S0103-50532009000900024

Brasil. Ministério da Saúde. AGÊNCIA NACIONAL DE VIGILANCIA SANITÁRIA. Farmacopeia Brasileira, volume I. 6ª Ed. Brasília, 2019.

Cao, B. et al. (2020) Um ensaio de lopinavir-ritonavir em adultos hospitalizados com Covid-19 grave . N. Engl. J. Med . 382 , 1787 - 1799 . https://doi.org/10.1056/NEJMoa2001282

Chaudhary, M., Kumar, N., Baldi, A., Chandra, R., Babu, M.A., Madan, J. (2020). Chloro and bromo-pyrazole curcumin Knoevenagel condensates augmented anticancer activity against human cervical cancer cells: design, synthesis, in silico docking and in vitro cytotoxicity analysis. Journal of Biomolecular Structure and Dynamics. 38:1, 200-218. DOI: 10.1080/07391102.2019.1578264

Dassault Systemes BIOVIA, 2017. Discovery Studio Modeling Environment, Release 2017. Dassault Systemes, San Diego.

Devpura, G.; Tomar, B.S.; Nathiya, D.; Sharma, A.; Bhandari, D.; Haldar, S.; Balkrishna, A.; Varshney, A. (2021) Randomized placebo-controlled pilot clinical trial on the efficacy of ayurvedic treatment regime on COVID-19 positive patients. Phytomedicine. 84, 153494. https://doi.org/10.1016/j.phymed.2021.153494.

Ding, T.; Zhao, T.; Li, Y.; Liu, Z.; Ding, J.; Ji, B.; Wang, Y.; Guo, Z. (2021). Vitexin exerts protective effects against calcium oxalate crystal-induced kidney pyroptosis in vivo and in vitro. Phytomedicine. 86, 153562. https://doi.org/10.1016/j.phymed.2021.153562.

Gazda, V. E.; Gomes-Carneiro, M. R.; Barbi, N. S.; Paumgartten, F. J. R.(2006) J. Ethnopharmacol. 105, 187.

He, J. D.; Wang, Z.; Li, S. P.; et al. (2016). Vitexin suppresses autophagy to induce apoptosis in hepatocellular carcinoma via activation of the JNK signaling pathway. Oncotarget. 7, 51, 84520–84532.

Holshue, M.L.; DeBolt, C.; Lindquist, S.; Lofy, K.H.; Wiesman, J.; Bruce, H.; Spitters, C.; Ericson, K.; Wilkerson, S.; Tural, A.; Diaz, G.; Cohn, A.; Fox, L.; Patel, A.; Gerber, S.I.; Kim, L.; Tong, S.; Lu, X.; Lindstrom, S.; Pallansch, M.A.; Weldon, W.C.; Biggs, H.M.; Uyeki, T.M.; Pillai, S.K. (2020) Washington State -nCo, V.C.I.T. First Case of 2019 Novel Coronavirus in the United States. N. Engl. J. Med. 382, pp. 929-936.

Hospital, A.; Andrio, P.; Fenollosa, C.; Cicin-Sain, D.; Orozco, M.; Gelpí, J. L. (2012) MDWeb and MDMoby: an integrated web-based platform for molecular dynamics simulations. Bioinformatics, v. 28, n.9, p. 1278-1279.

Hu, K.; Guan, W.J.; Bi, Y.; Zhang, W.; Li, L.; Zhang, B.; Liu, Q.; Song, Y.; Li, X.; Duan, Z.; Zheng, Q.; Yang, Z.; Liang, J.; Han, M.; Ruan, L.; Wu, C.; Zhang, Y.; Jia, Z.H.; Zhong, N.S. (2020). Efficacy and Safety of Lianhuaqingwen Capsules, a repurposed Chinese Herb, in Patients with Coronavirus disease 2019: A multicenter, prospective, randomized controlled trial. Phytomedicine: international journal of phytotherapy and phytopharmacology. Article 153242

Jacob, K.S., Ganguly, S., Kumar, P., Poddar, R., Kumar, A. (2017). Homology model, molecular dynamics simulation and novel pyrazole analogs design of Candida albicans CYP450 lanosterol 14 α-demethylase, a target enzyme for anti-fungal therapy. Journal of Biomolecular Structure and Dynamics. 35 (7), pp. 1446-1463

Jaffe, M.: J. (1886). Phisiol. Chem. 10:381.

Krishnan, M; Kang, S. C. (2019) Vitexin inhibits acrylamide-induced neuroinflammation and improves behavioral changes in zebrafish larvae. Neurotoxicology and Teratology. 74, 106811.

Lan, J., Ge, J., Yu, J. et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. Nature 581, 215–220 (2020). https://doi.org/10.1038/s41586-020-2180-5

Lyu S.Y.; Rhim J.Y.; Park W.B. (2005) Antiherpetic activities of flavonoids against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in vitro. Arch Pharm Res. 28: 1293-301.

Mhatre, S.; Srivastava, T.; Naik, S.; Patravale, V. (2021) Antiviral activity of green tea and black tea polyphenols in prophylaxis and treatment of COVID-19: A review. Phytomedicine. 85, 153286. https://doi.org/10.1016/j.phymed.2020.153286.

Mors, W.B.; Rizzini, C.T.; Pereira, N.A. (2000). Medicinal Plants of Brazil Reference Publications, Michigan pp. 297.

Moura, WS, Souza, SR, Campos, FS, Cangussu, ASR, Santos, EMS, Andrade, BS, Viana, KF, Haddi, K., Oliveira, EE, Nascimento, VL, Aguiar, RWS, 2020. Antibacterial activity of *Siparuna guianensis* essential oil mediated by impairment of membrane permeability and replication of pathogenic bacteria. Ind. crops prod. 46, 1-9. https://doi.org/10.1016/j.indcrop.2020.112142

Negri, G., Santi, D., Tabach, R. 2012. Chemical composition of hydroethanolic extracts from *Siparuna guianensis*, medicinal plant used as anxiolytics in Amazon region. Braz. J. Pharmacog. 5:1024- 1034.

Oliveira, M.S., Cruz, J.N., Silva, S.G., Costa, W.A., Sousa, S.H.B., Bezerra, F.W.F., Teixeira, E., Silva, N.J.N., Andrade, E.H.A., Neto, A.M.J.C., Carvalho, R.N. (2019). Phytochemical profile, antioxidant activity, inhibition of acetylcholinesterase and interaction mechanism of the major components of the Piper divaricatum essential oil obtained by supercritical CO2. The Journal of Supercritical Fluids. 145, 74-84. https://doi.org/10.1016/j.supflu.2018.12.003

Oliveira, M.S., da Cruz, J.N.; Almeida da Costa, W.; Silva, S.G.; Brito, M.d.P.; de Menezes, S.A.F.; de Jesus Chaves Neto, A.M.; de Aguiar Andrade, E.H.; de Carvalho Junior, R.N. (2020). Chemical Composition, Antimicrobial Properties of Siparuna guianensis Essential Oil and a Molecular Docking and Dynamics Molecular Study of its Major Chemical Constituent. *Molecules. 25*, 3852. https://doi.org/10.3390/molecules25173852

Reitman, S; Frankel, S. (1957) Am. J. Clin. Path. 28:56.

Sachdeva, C.; Wadhwa, A.; Kumari, A.; Hussain, F.; Jha, P.; Kaushik, N.K. (2020). OMICS: A Journal of Integrative Biology. 568-580.http://doi.org/10.1089/omi.2020.0071

Sancesario, G. M.; Bernardini, S. (2018) Alzheimer's disease in the omics era. Clinical Biochemistry. 59, 9–16.

Sanner, M.F., 1999. Python: a programming language for software integration and development.

Schrodinger, L.L.C., 2018. The PyMOL Molecular Graphics System, Version 2.0.

Sehrawat, N.; Upadhyay, S.K.; Sharma, A.K. et al.(2021) Papel Renoprotetor Emergente do Flavonóide Naringina: Status Farmacêutico Atual e Perspectivas Futuras. Curr Pharmacol Rep. 7, 96-101. https://doi.org/10.1007/s40495-021-00256-7

Senegaglia, A.C.; Rebelatto, C.L.K.; Franck, C.L.; Lima, J.S.; Daga, D.R.; Shigunov, P.; Azambuja, A.P.; Bana, E.G.; Marsaro, D.B.; Schaidt, B.; Micosky, A.; Jamur, V.R.; Vaz, I.M; Schluga, Y.; Leite, L.M.B.; Ribeiro, L.L.; Correa, A.; Brofman, P.R.S. (2021). Use of tocilizumab and mesenchymal stromal cells in the treatment of severe covid-19 - a case report. Cytotherapy. 23, 4, Supplement, 38-39.https://doi.org/10.1016/j.jcyt.2021.02.111.

Singer JM, Plotz CM, Pader E, Elster SK. (1957)The latex fixation test. III Agglutination test for c-reactive protein and coparison with the capillary precioitin method. Am J Clin Pathol. 28:611

Shi, N.; Guo, L.; Liu, B.; Bian, Y.; Chen, R.; Chen, S.; et al. (2019). Efficacy and safety of Chinese herbal medicine versus Lopinavir-Ritonavir in adult patients with coronavirus disease 2019: A non-randomized controlled trial. Phytomedicine. 81, 153367. https://doi.org/10.1016/j.phymed.2020.153367.

Silva, F. de A. S. e.; Azevedo, C. A. V. de. (2016) The Assistat Software Version 7.7 and its use in the analysis of experimental data. Afr. J. Agric. Res.; 11, 39, 3733-3740. Doi: 10.5897/AJAR2016.11522

Soares, I. M., Fátima Ribeiro, M., Costa, O. J., Souza, É. E., Aguiar, A. A., Santos Barbosa, R., Costa, T. Application of a degreasing process and sequential ultrasound-assisted extraction to obtain phenolic compounds and elucidate of the potential antioxidant of *Siparuna guianensis* Aublet. Journal of Medicinal Plants Research. Vol. 11(21), pp. 357-366, 3 June, 2017.

Trott, O., Olson, A.J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. J. Comput.Chem. 31, 455–461. https://doi.org/10.1002/jcc.21334.

Wang, Y.; Zhang, D.; Du, G.; Du, R.; Zhao, J.; Jin, Y.; Fu, S.; Gao, L.; Cheng, Z.; Lu, Q.; Hu, Y.; Luo, G.; Wang, K.; Lu, Y.; Li, H.; Wang, S.; Ruan, S.; Yang, C.; Mei, C.; Wang, Y.; Ding, D.; Wu, F.; Tang, X.; Ye, X.; Ye, Y.; Liu, B.; Yang, J.; Yin, W.; Wang, A.; Fan, G.; Zhou, F.; Liu, Z.; Gu, X.; Xu, J.; Shang, J.; Zhang, Y.; Cao, L.; Guo, T.; Wan, Y.; Qin, H.; Jiang, Y.; Jaki, T.; Hayden, F.G.; Horby, P.W;. Cao, B.; Wang, C. (2020). Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. Lancet. 395, 1569-1578. Doi: 10.1016/S0140-6736(20)31022-9

Wang, M.; Cao, R.; Zhang, L.; Yang, X.; Liu, J.; Xu, M, et al. (2020). Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res. 30: 269–271. doi: https://doi.org/10.1038/s41422-020-0282-0

Yahaya, M.A.F. et al.(2020). Possible epigenetic role of vitexin in regulating neuroinflammation in Alzheimer's disease. J. Immunol. Res. 9469210. Doi: 10.1155/2020/9469210

Yan, B.; Jiang, Z.; Yuan, J.; Li, M.; Zeng, J.; Tang, J.; Lu, Z.K.; Ding, H.; Xia, J.; Wang, Q.; Wang, J.; Xie, H.; Li, W.; Zhang, N.; Li, H.; Sang, X.; Wu, L.; Xiong, X.; Tang, S.; Li, Y.; Tao, M.; Xie, C.; Yu, S. (2021) Effects and safety of herbal medicines among community-dwelling residents during COVID-19 pandemic: A large prospective, randomized controlled trial (RCT). Phytomedicine. 85, 153403. https://doi.org/10.1016/j.phymed.2020.153403.

Zandi, K., Teoh, BT., Sam, SS. et al. (2011) Antiviral activity of four types of bioflavonoid against dengue virus type-2. Virol J. 8, 560. https://doi.org/10.1186/1743-422X-8-560

Zhou, F.; Yu, T.; Du, R.; Fan, G.; Liu, Y.; Liu, Z.; Xiang, J.; Wang, Y.; Song, B.; Gu, X.; Guan, L.; Wei, L.; Li, H.; Wu, X.; Xu, J.; Tu, S.; Zhang, Y.; Chen, H.; Cao, B. (2020). Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet, 395, 1054-1062.

Zhou, S.; Feng, J.; Xie, Q.; Huang, T.; Xu, X.; Zhou, D.; Zhang, W.; Sun, S.; Liu, X.; Wu, X.; Che, J.; Fan, T.; Zou, D.; Wang, J.; Zhan, D.; Peng, D.; Feng, Y.; Yu, G.; Yuan, Z.; Fang, B. (2021) Traditional Chinese medicine shenhuang granule in patients with severe/critical COVID-19: A randomized controlled multicenter trial. Phytomedicine. 89, 153612. https://doi.org/10.1016/j.phymed.2021.153612.

Annexes

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Antibacterial activity of *Siparuna guianensis* essential oil mediated by impairment of membrane permeability and replication of pathogenic bacteria

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ABSTRACT

The increasing prevalence of resistance to conventional antibiotics in pathogenic bacteria has demanded faster development of novel sources of antibacterial agents. In this context, biological activities shown by natural compounds have received particular attention. One of the alleged backslashes for these alternative bactericidal tools is the current knowledge gap regarding their action mechanisms. Here, we evaluated the activity of essential oil extracted of Negramina, *Siparuna guianensis*, plants against pathogenic bacteria *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*. We evaluated cytotoxic effects on bacterial cells and the action mechanisms of the essential oil. The gas chromatography analysis revealed that β-Myrcene (39.68%), epicurzerenone (18.16%) and germacrenes D (14.34%) and B (2.93%) are the major components of the *S. guianensis* essential oil. Interestingly, the essential oil exhibited toxicity against all pathogenic bacteria without affecting the human monocytic THP-1 cell line. This antibacterial activity resulted from strong bacterial growth inhibition and deregulation of bacterial cell wall permeability with increased nucleotides and K^+ ions leakage. Furthermore, our molecular docking predictions indicated high affinity between some essential oil major components Germacrene B and active sites of bacterial DNA and RNA polymerases, which indicates possible impairments on the pathogenic bacteria cell replication processes.

1. Introduction

Pathogenic bacteria like *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes* are ubiquitous microorganisms that survive under a variety of environmental conditions. Their importance derives from the health problems that they pose being agents of numerous diseases in humans and other warm-blooded animals (Oliveira et al., 2019; Rahman et al., 2018). Traditionally, the control of pathogenic bacteria is achieved using antimicrobial agents including antibiotic drugs and disinfectant agents with bactericidal effects.

Bactericidal agents induce bacterial cell death targeting a diverse set of biomolecules responsible for essential cellular processes. Exposure to bactericidal antibiotics has been shown to kill bacteria by inhibiting these cellular processes and by activating cellular response pathways that contribute to cell death (Kohanski et al., 2010). Some of these processes, include DNA and RNA synthesis (DNA and RNA polymerases), protein synthesis (ribosomes), cell wall homeostasis

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(transglycosylases and peptidoglycan building blocks), as well as modulation of DNA topology (DNA topoisomerases) and penicillin-binding proteins (Kohanski et al., 2010; Daly et al., 2000; Sanyal and Doig, 2012).

On the other hand, bacterial resistance to antibiotics is becoming a global health issue (Scarafile, 2016) and the emergence of resistant bacteria is causing problems for both the treatment of patients and infections' control. In fact, some pathogenic bacteria have become resistant to entire classes of antibiotics, as in the case of *E. coli* resistance to ciprofloxacin (Wang et al., 2019), *S. aureus* resistance to methicillin (Krishnamoorthy et al., 2018), *P. aeruginosa* resistance to ampicillin (El-Banna et al., 2019) and *S. pyogenes* resistance to tetracycline (Kalumbi, 2019). Because of this rise and widespread of bacteria resistant to several drugs, bacterial infections have become major health challenges, generating increased interest in the search for and development of new antimicrobial agents.

Since antiquity, plants and their derivatives, including essential oils (EOs), have been used in folk medicine. Besides their important roles in the protection of plants, EOs present a broad range of secondary metabolites that are frequently reported to inhibit or slow the growth of bacteria, yeasts and molds (Nazzaro et al., 2013). Many studies have described the antimicrobial effects of EOs against both gram-positive bacteria such as *S. aureus* and *S. pyogenes* and gram-negative bacteria such as *E. coli* and *P. aeruginosa* (Lee et al., 2014; Tao et al., 2019; Heller and Spence, 2019).

The activity of EOs is known to depend on their chemical composition. The antimicrobial activity of EOs has a variety of targets in the bacterial cell membrane, and cytoplasm. They can disturb the cell permeability, damage the cell wall and the membrane proteins leading to leakage of the cell contents and alterations of the intracellular and external ATP balance (Nazzaro et al., 2013). EOs can also induce complete morphology change of the bacterial cells through alterations in its structure and functionality (Nazzaro et al., 2013).

Siparuna guianensis, popularly known as *Negramina,* is a Neotropical plant belonging to the Siparunaceae family that is recently drawing increasing interest for the aromatic, medicinal and biological properties of its essential oils and extracts. Products derived from its leaves, bark, and flowers have been widely used in folk medicine to treat sinusitis, fever, rheumatism, migraine, influenza, and body aches (Renner and Hausner, 2005; Valentini et al., 2010). Recently, the essential oil of this plant species was also explored for its potential in insect pest management with promising results (Ferreira et al., 2017b, 2019; Lourenço et al., 2018). Moreover, compounds found in its essential oil including β-myrcene (Basera et al., 2019), germacrene D (Elshafie et al., 2019), germacrene B (Runyoro et al., 2010) and 2-undecanone are reported to have substantial antimicrobial potentials (Boudjema et al., 2018).

In the present study, we firstly investigated the antibacterial activity of the EOs of *S. guianensis* against four species of pathogenic bacteria (i.e., *E. coli*, *S. aureus*, *P. aeruginosa* and *S. pyogenes*). Then, we assessed the cytotoxicity of *S. guianensis* essential oil both to bacterial cells and to human TPH-1 cells. Finally, we investigated the mechanisms behind the antibacterial activity at cells wall permeability level and we analyzed *in silico* molecular docking interactions of *S. guianensis* EO major compounds with bacterial DNA and RNA polymerases.

2. Materials and methods

2.1. Bacterial species

Antibacterial activity of *S. guianensis* EO compounds were evaluated against four bacterial species from the American Type Culture Collection (ATCC, Rockville, MD, US): *S. aureus* (ATCC 25,923), *S. pyogenes* (ATCC 19,615), *E. coli* (ATCC 35,218) and *P. aeruginosa* (ATCC 2753). All strains were maintained on slopes of Skim Milk (Oxoids), stored at −20 °C, and subcultured two days before the assays to prevent morphological and metabolic transformations (Arunachalam et al., 2016). Each strain was inoculated into 10 mL Mueller-Hinton broth and incubated at 37 °C under agitation of 200 rpm (Shaker Marconi – Mod. MA420, Brazil). After a 24-h incubation period, the suspensions were dispersed in sterile saline in glass tubes and stirred in a vortex until obtaining turbidity equivalent to the tube number 0.5 of the nephelometric McFarland scale (Lennet et al., 1985).

2.2. Plant material, essential oil extraction and gas chromatography–mass spectrometry (GC–MS) analysis

The leaves of *Siparuna guianensis* were collected and extraction of its essential oil was performed according to the methods described by Ferreira et al. (2017b) and, taxonomic identification was made by herbarium specialists from the Federal University of Tocantins (Porto Nacional-TO, Brazil - 10,496). The present investigation was registered in Brazil – SISGEN under number A7CAD12. The essential oils were extracted from *S. guianensis* leaves using the steam distillation method as described by (Aguiar et al., 2015; Lourenço et al., 2018). The gas chromatography–mass spectrometry (GC–MS) analysis was performed according to the methods described in Ferreira et al. (2017b), being the analysis of *S. guianensis* EO performed on the Chemito 8510 GC instrument (Chemito Technologies Pvt. Ltd, Mumbai, India) at the analytical center IQ-USP (São Paulo-SP, Brazil).

2.3. Antibacterial assays

2.3.1. Minimum inhibitory concentration (MIC)

To determine the minimum inhibitory concentration (MIC) of *S. guianensis* EO to *E. coli*, *S. pyogenes*, *P. aeruginosa* and *S. aureus*, the agar diffusion principle was used, according to the protocol recommended by Clinical and laboratory standards institute/NCCLS, 2005 with some adaptations. With suitably sterilized swabs, the bacteria were homogeneously inoculated at concentrations of approximately 1.5×10^8 CFU / mL (Mac Farland 0.5 scale) in petri dishes containing Mueller Hinton Agar (MHA). Following the inoculation, concentrations of 0.87, 1.30, 1.70, 2.12, 17, 34, 68, and 102 µg/mL of the essential oil of *S. guianensis* were placed in 6-mm diameter wells as described by Ostrosky et al. (2008). The lowest concentration of EO that inhibited the growth of the bacteria analyzed was considered the MIC.

2.3.2. Bacterial growth curve

The growth curves of *E. coli*, *S. pyogenes*, *P. aeruginosa* and *S. aureus* were evaluated following the method of Peyret et al. (1990) and Sheoran and Tiwari (2019). Briefly, bacterial samples were inoculated into Mueller Hinton broth and cultured for eight hours under stirring at 200 rpm and 37 °C, using with initial OD_{600} 0.02. Then, increasing concentrations of *S. guianensis* EO (0.87, 1.30, 1.70, and 2.12 µg/ml) were homogenized using 200 µL of dimethylsulfoxide (DMSO) in 1 mL of the standardized (Mac Farland 0.5 scale) inoculum of bacterial samples and added to 20 ml of Mueller Hinton broth. The preparations were shaken (200 rpm) at 37 °C until measurements of optical densities. The control consisted of only 1 mL of standardized bacteria in 20 ml of Mueller Hinton broth and 200 µL of DMSO. The optical density readings at 600 nm were performed using a spectrophotometer (Quimis) at five intervals corresponding to 120, 210, 330, 390, and 430 min, after inoculation (Heller and Spence, 2019).

2.3.3. Intracellular K^+ efflux

The potassium efflux effect was measured according to the method of Arunachalam et al. (2016) and Oliveira et al. (2015) with adaptations. Initially, *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* cells were cultured overnight at 37 °C. The cells were washed and resuspended at a concentration of 1×10^7 cells/mL in phosphate buffered saline (PBS) of pH 7.2. Subsequently, 1 mL of the bacterial suspensions containing *S. guianensis* EO and other rifamycin SV sodium (RSS) was incubated at 37 °C for different times. Bacterial strains incubated with PBS alone

were used as controls. After centrifugation, the amounts of released K^+ in the supernatants were measured using a Microprocessor Flame Photometer (Quimis, Model Q498M2, São Paulo, SP, Brazil).

2.3.4. Nucleotide leakage

The nucleotide leakage was performed according to Arunachalam et al. (2016) with adaptations. Initially, *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* cells in logarithmic growth phase were washed and resuspended in 10 mM PBS (pH 7.2). The bacterial strains were incubated with *S. guianensis* EO and RSS for different times. Bacterial strains incubated with PBS alone served as the controls. The mixture absorbance was determined using a spectrophotometer at 260 nm (Shimadzu UV-1800, Vernon Hills, IL, US).

2.4. Cytotoxicity of S. Guianensis EO to human TPH-1 cells

Essential oil of *S. guianensis* was dissolved in DMSO and diluted in RPMI culture medium (Sigma-Aldrich™) to form a stock solution. The cell viability test was performed using TPH-1 cells (ATCC® TIB-202™). Cytotoxicity was measured using the microplate dilution method. Once attached, the culture medium was removed and sample solutions were added at concentrations of 0.87, 1.30, 1.70, and 2.12 µg/mL. The final volume in each well was 100 µl and the quantity of cells present in each well was 1×10^4 cells. The plates were incubated for 48 h at 37 °C in a humidified atmosphere with 5% CO_2. Next, 100 µl of MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) was added and incubated for 4 h. Readings of absorbance at 540 nm were performed using a microplate spectrophotometer (Quimis). The assays were performed in triplicate and the results of the absorbances for each concentration were calculated according to the growth control. The CC_{50} (cytotoxic concentration at which 50% of the cells are viable) was calculated using dose-response graph nonlinear regression (Pillay et al., 2007). The cytotoxic assays were tested using ANOVA with a significance level of 5% by the Tukey method using OriginPro 8 software (OriginLab, 2007).

To visualize the potential cytopathic effects of *S. guianensis* EO on human monocytic cells, THP-1 cells were incubated with respective concentrations of *S. guianensis* in RPMI medium. Negative controls without the addition of the essential oil were used. After 48 h, 10 µL of Hoechst 33,342 were added and incubated at 37 °C for 15 min. The supernatants were discarded, and the wells were washed with PBS, followed by addition of 5 µL of propidium iodide (PI) for 20 min. Subsequently, the wells were washed with PBS and the circular coverslips containing the labeled cells were fixed with 3% formalin and cell viability was analyzed using a fluorescence microscope. Viable cells were indicated by blue fluorescence and non-viable cells were indicated by red fluorescence.

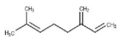
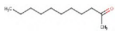
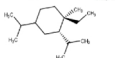
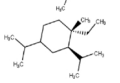
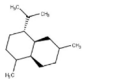
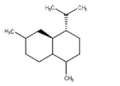
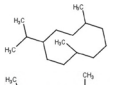
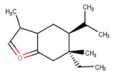
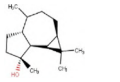
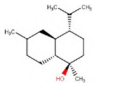
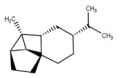
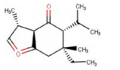
2.5. In silico studies of the interaction between the S. Guianensis EO molecules and the receptor bacterial DNA and RNA polymerases

Amino acid sequences of DNA and RNA polymerases from *E. coli*, *S. pyogenes*, *P. aeruginosa* and *S. aureus* were obtained from the National Center for Biotechnology Information (NCBI) database. 3D structures of both proteins were constructed by homology modeling approach with The Swiss Model Workspace (https://swissmodel.expasy.org/), after the selection of its respective templates using BLASTp tool. The templates were downloaded from The Protein Databank (https://www.rcsb.org/), considering quality parameters as experimental method, resolution and R-value, as well as its complexing with a ligand. To check protein structure crashes and amino acid positioning in the active site, we used the Swiss model (Waterhouse et al., 2018). Validation of the generated models was performed by inspection of the Ramachandran plots (Ramachandran and Sasisekharan, 1968;Haas et al., 2018), in which it was possible to analyze the distribution of the torsion angles of the backbone ϕ and ψ responsible for the stereochemical quality of the protein studied as well as the QMEAN factor (Benkert et al., 2011).

Nine docking positions were generated for each ligand interacting with bacterial DNA and RNA polymerases, returning affinity energy values (kcal/mol) using the AutoDock Vina (Trott and Olson, 2010) in the docking calculations, initially the *Siparuna guianensis* molecules designed with Marvin Sketch 18.10 (ChemAxon) and receptors and ligands prepared for the molecular docking process using Autodock Tools 1.5.7 (Sanner, 1999), according to the methodology proposed by Borges et al. (2019).

The docking position results were analyzed using PyMOL 2.0 (Schrodinger, 2018) and Discovery Studio 4.5 (Dassault Systemes BIOVIA, 2017) for selecting the best position for each ligand inside the protein target using the parameters proposed by Borges et al. (2019).

Table 1
Chemical composition of *Siparuna guianensis* essential oil.

Compound	Molar mass	Molecular formula	Chemical structure	%	Ric*
β-Myrcene	136.2	$C_{10}H_{16}$		39.67	986
2-Undecanone	170.2	$C_{11}H_{22}O$		6.25	1251
γ-Elemene	204.3	$C_{15}H_{24}$		3.5	1439
Elixene	204.3	$C_{15}H_{24}$		3.5	1435
γ-Muurolene	204.3	$C_{15}H_{24}$		1.34	1441
δ-Cadinene	204.3	$C_{15}H_{24}$		1.25	1478
Germacrene D	204.3	$C_{15}H_{24}$		14.34	1528
Curzerene	216.3	$C_{15}H_{20}O$		4.91	1542
Spathulenol	220.3	$C_{15}H_{24}O$		1.03	1547
α-Cadinol	222.3	$C_{15}H_{26}O$		1.32	1592
Germacrene B	204.3	$C_{15}H_{24}$		2.93	1613
Epicurzerenone	230.3	$C_{15}H_{24}O_2$		18.16	1611
Not identified (%)	–	–	–	1.80	–

* Ric = Retention index calculated.

3. Results

3.1. Chemical composition of the EO from S. Guianensis

The composition and identification of the main compounds present in *S. guianensis* EO in GC–MS analyses revealed 12 compounds (Table 1). The major components from the leaves were β-myrcene with 39.67%, germacrene D with 14.34% and epicurzerenone with 18.16%. We also detected germacrene B at 2.93%.

3.2. Determination of minimum inhibitory concentration (MIC)

The MIC results among the tested concentrations of *S. guianensis* EO are presented in Table 2. The low concentrations for the MIC suggest

Table 2
Antimicrobial activity of the *S. guianensis* EO expressed as minimum inhibitory concentration (MIC).

Bacteria	GRAM	MIC value (μg/mL)
S. aureus	+	1.30 a
E. coli	–	0.87 b
P. aeruginosa	–	0.87 b
S. pyogenes	+	0.87b

Lowercase letters for columns indicate significant differences between MIC values for the different concentrations of *S. guianensis* EO, according to Tukey's test ($\alpha = 0.05$).

that the bacterial species used are highly susceptible to the effect of *S. guianensis* EO, with MIC values ranging from 0.86 μg/mL (*E. coli*, *S. pyogenes*, *P. aeruginosa*) to 1.30 (*S. aureus)*.

3.3. Growth curve of the bacteria against S. Guianensis EO

Optical absorbances of bacterial cultures over time in the presence of increasing concentrations of *S. guianensis* EO (Fig. 1) showed that even the lowest concentration (0.87 μ /mL) was extremely toxic and inhibited cell growth of *E. coli, P. aeruginosa* and *S. pyogenes.* The same concentration slowed the cell growth of *S. aureus* with OD reaching only 0.332 after 430 min. Higher concentrations (1.70 and 2.12 μg/ml) completely inhibited the growth.

3.4. K^+ efflux induced from S. Guianensis EO

Potassium efflux independently increased during the contact period of the S. guianensis EO with bacteria strains. We observed that the maximum value was found at the 2nd hour for *S. aureus* (4.3 ppm), at the 3rd hour for *S. pyogenes* (4.8 ppm), and 4th hour for *E. coli* and *P. aeruginosa* (4.4 and 5.4 ppm, respectively), while the control showed a slight variation of only 0.3 ppm throughout the experiment. For the RSS antibiotic, we found that *S. aureus* had its highest value at the 3rd hour (4.4 ppm) and *E. coli*, *S. aureus* and *S. pyogenes* had higher values for potassium efflux at the 4th hour (3.7, 4.2 and 4.2 ppm, respectively) (Fig. 2).

3.5. Nucleotide leakage

There was considerable nucleotide leakage in all bacteria tested, starting from the 2nd hour with the greatest effect for the test in the presence of *S. guianensis* EO with *E. coli* (0.477), *P. aeruginosa* (0.575), *S. aureus* (0.568) and *S. pyogenes* (0.522), when compared to the RSS antibiotic that showed values of 0.376, 0.392, 0.157 and 0.257, respectively (Fig. 3). There was very little variation of the control throughout the experiment.

3.6. Cytotoxicity of S. Guianensis EO to TPH-1 cells (human monocytic cells)

We tested toxicity of *S. guianensis* EO in human monocytic cells (TPH1 cell line) at 0.87, 1.30, 1.70, and 2.12 μg/ml. None of these concentrations caused significantly more toxicity than controls (Fig. 4). The concentrations of *S. guianensis* EO that showed inhibitory effects on *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* bacterium were not toxic for TPH1 cells.

Similar to the results of the MTT cytotoxicity test, cell viability analyses using immunofluorescence indicated that no concentration of *S. guianensis* EO generated significant toxic effects (Fig. 4). In all analyzed fields, there was a predominance of THP-1 cells with blue fluorescence.

3.7. Interaction of S. Guianensis EO molecules and bacterial DNA and RNA polymerases

According to the results obtained from bacterial bioassays, our

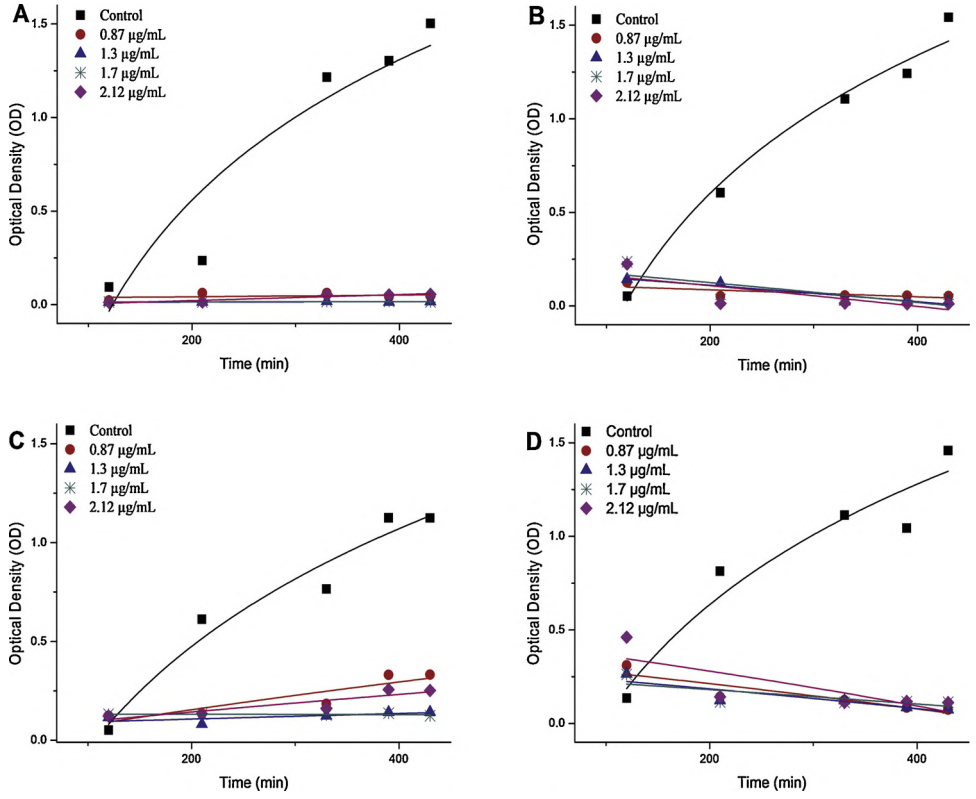


Fig. 1. Effect of EO *S. guianensis* on the determination of growth curves (A) *E. coli*, (B) *P. aeruginosa*, (C) *S. aureus* and (D) *S. pyogenes.*

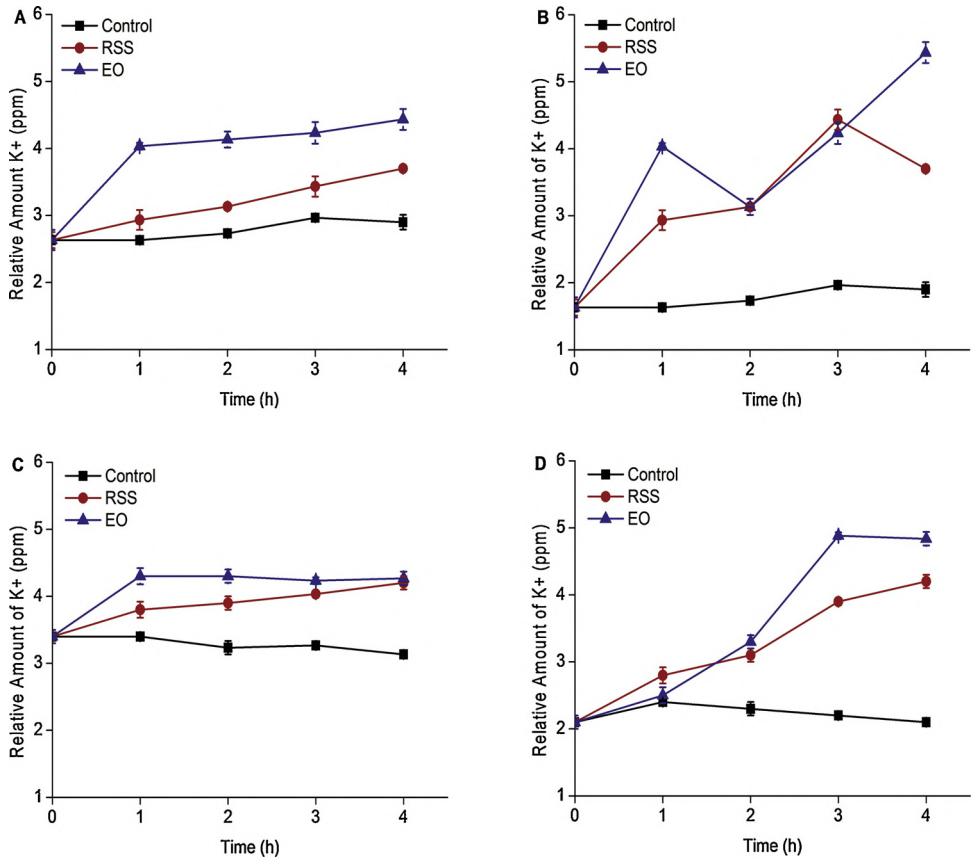


Fig. 2. Effect of MIC of EO *S. guianensis* on the amount K^+ release from (A) *E. coli*, (B) *P. aeruginosa*, (C) *S. aureus* and (D) *S. pyogenes*. Each value represents mean ± SD; three independent experiments.

hypothesis is that main compounds present in S. guianensis EO could interact with the enzymes' DNA and RNA polymerases of these bacteria, inhibiting replication. Table 3 shows the selected templates for homology modeling, highlighting the identities and the validation results with the corresponding Ramachandran favored values.

The compounds present in S. guianensis EO complexed with the different receptors and formed various types of interactions with varying affinity energies as indicated by the docking assays performed (Table 4). Besides all complexed ligands, germacrene B presented better affinity energy with *E. coli*, *P. aeruginosa* and *S. aureus* DNA and RNA polymerases (Figs. 5A, B, C, 6 A, B and C, respectively), as well as complexed with the RNA polymerase of *S. pyogenes* (Fig. 6D).

Germacrene B complex with E. coli DNA polymerase showed interactions with active site amino acids and the ligand: alkyl-type interactions with PHE228, MET314 and PRO710 and van der Waals interactions with ASP156 ILE157, GLU158, THR159, TRP223, ASN224, LYS305 and ARG313 (Fig. 5D). For the RNA polymerase, we found alkyl interactions with PHE14 and MET11, and van der Waals interactions with ASN10 and GLN18 (Fig. 6E). The complex formed between germacrene B and *P. aeruginosa* DNA polymerase presented alkyl-type interactions with VAL427, TYR430 and PHE436, and van der Waals interactions with TYR590 (Fig. 5E). Additionally, germacrene B complexed with *P. aeruginosa* showing alkyl interactions with ALA157, PHE169, PHE171, VAL177, ALA189, PHE207 and ILE331, and van der Waals interactions with PHE140, GLU170 and LEU193 (Fig. 6F).

DNA polymerase of *S. aureus* receptors complexed with germacrene B showing alkyl-type interactions with at LEU583, LEU584, TYR588, PRO627, VAL628 and ARG629, as well as Van deer Walls with GLN580, ASN625, ILE626, LEU630, GLU632 and GLY633 (Fig. 5F). On the other hand, the complex with RNA polymerase presented only one alkyl interaction with PHE14, and van der Waals interactions with ASN10, MET11 and GLN18 (Fig. 6G).

Due to low identity between target and templates, it was not possible to construct the DNA polymerase structure from *S. pyogenes*. However, we constructed a reliable 3D structure of RNA polymerase, and its interaction with germacrene B showed alky interactions with TYR62, TYR65, PRO70 and VAL172, and van der Waals interactions with VAL173 (Fig. 6H).

For all targets, we performed a re-docking with rifamycin SV sodium as a positive control. For all docking results, germacrene B occupied the same region of the ligand control (Supplementary Fig. 1).

4. Discussion

The *Siparuna guianensis* EO had β-myrcene as its primary component, this is a bactericidal molecule for human pathogens such as *Streptococcus pneumonia*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* (Skocibusic et al., 2004). The EO also contains germacrenes D and B, found also in the EO of *Copaifera officinalis* that has bactericidal activity for *S. aureus* and *E. coli* (Andrade et al., 2013). Other authors have found various chemical elements in varying proportions than those found in the present study (Andrade et al., 2013; Ferreira et al., 2017a), suggesting that the composition of *S. guianensis* EO depends on its location, as well as on the period in which the plant was collected and its oil was extracted.

Antibacterial activity of compounds is considered significant when the MIC is below 10 μg/mL (Omosa et al., 2016). This suggests that MIC values ranging from 0.86 to 1.30 μg/ml for *S. guianensis* EO against the bacteria used in this study were effective. In the EO of *Rosmarinus officinalis* obtained by hydrodistillation, the MIC value was 3.75 μg/mL for *S. aureus* and 7.5 μg/mL for *E. coli* (Probst, 2012). In assays using *A. herba-alba* performed by Sbayou et al. (2014), the MIC value was

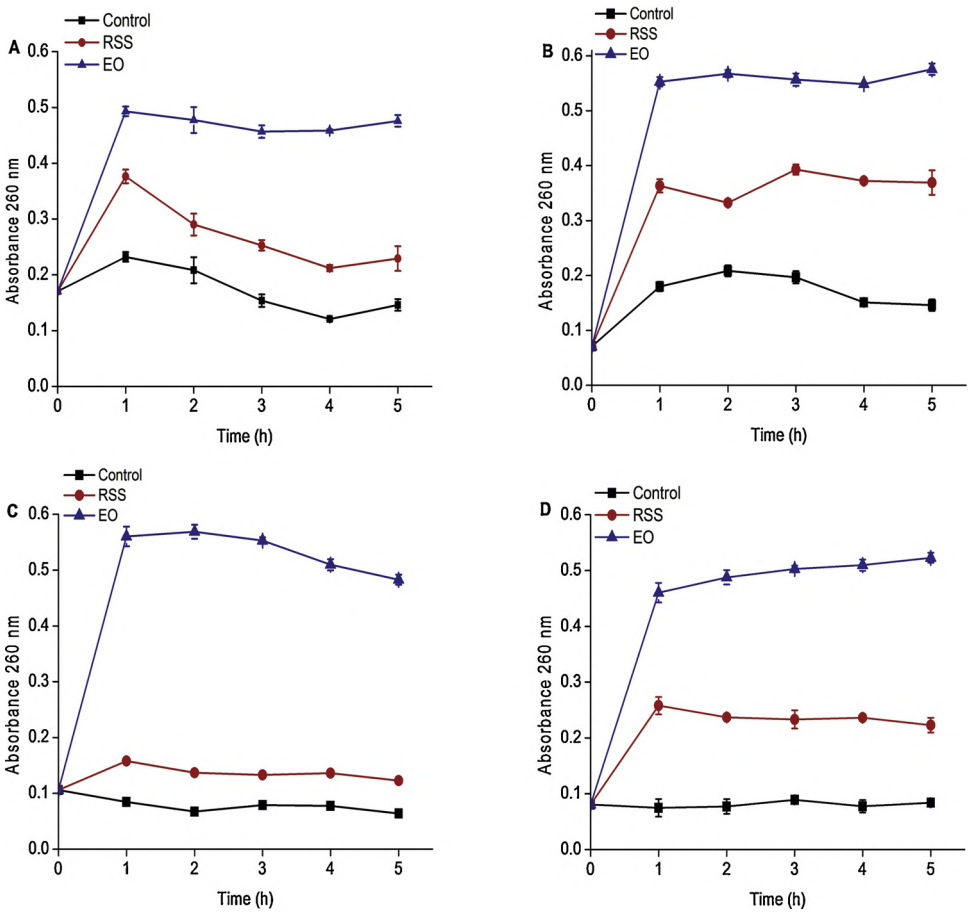


Fig. 3. Effect of MIC of EO *S. guianensis* on the amount total nucleotide release from (A) *E. coli*, (B) *P. aeruginosa*, (C) *S. aureus* and (D) *S. pyogenes*. Each value represents mean ± SD; three independent experiments.

against *E. coli* was 1.25 µg/mL. Mighri et al. (2010) reported MIC of 0.62 mg/mL against *S. aureus*, a value higher than found in the present study, where the essential oil of *S. guianensis* showed strong antibacterial effects against both gram-positive and gram-negative bacteria.

We observed that while the control samples showed expected growth rates, the samples treated with OE showed growth inhibition. The EO prevented bacterial replication at all concentrations tested. According to Cui et al. (2018), OD values are influenced by viable bacterial cell count; this suggests that, after treatment with *S. guianensis* EO, we observed from the constant OD that there was no increase in the number of viable cells.

The decrease in OD values for the various bacteria in contact with *S. guianensis* EO at various time points of the experiment suggests bactericidal effects, as proposed by Nocchetti et al. (2013). In the same sense, Bachir and Benali (2012) observed inhibition of growth of *S. aureus* and *E. coli* caused by *E. globulus* EO measured in terms of optical density at various concentrations of essential oil, and *S. aureus* growth was totally inhibited when 50 µL of the oil was used within 2 h of exposure. At 100 µL of the oil, 10 min exposure inhibited the growth of the bacteria for all amounts of broth dilution.

Comparatively speaking, all the concentrations used in this work were low, compared to those used in other works, such as those of Xu et al. (2018), who observed inhibition halos at 12.7 µg/mL of *Artemisa Asian* EO against *S. aureus*, 14.3 µg/mL against *S. pyogenes*, and 9.2 µg/mL against *P. aeruginosa*, which demonstrates the good effect provided by the EO of *S. guianensis*.

We can also note that the EO increased the bacterial cell permeability, this phenomenon is identified by analysis efflux of K^+ and extracellular nucleotide leakage. Other authors also observed the increased permeability, causing membrane damage to bacteria using different essential oils (Lin et al., 2018;Cui et al., 2019).

According to Bajpai et al. (2013) EO components cause a loss of their integrity thus correlating to the leakage of various substances such as ions, ATP, nucleic acids and amino acids. The gram-negative bacterial cell wall is made up of lipopolysaccharides that block the penetration of hydrophobic components while the single membrane of gram-positive bacteria is considerably more accessible to permeation by hydrophilic EO components at target sites (Bezic et al., 2003) however, no significant differences were observed for *S. guianensis* EO action in these gram-positive or gram-negative bacteria.

Transport of K^+ ions is extremely important for bacterial pathogenesis via regulation of cytoplasmic and cellular pH, primarily mediated by efflux control, as explained by Roosild et al. (2010). Unregulated leakage of ions such as potassium suggests damage that EO causes to the cytoplasmic membrane, allowing exposure of vital intracellular materials.

We note that *S. guianensis* EO shows better results than the reference RSS antibiotic, it shows its ability to increase the permeability of the membrane, where we have an increase of K^+ efflux concentration of approximately 3 ppm for *P. aeruginosa* and *S. pyogenes*, higher than RSS at approximately 1 ppm after 4 h in analysis.

We observed that the nucleotide leakage analysis showed that *S. guianensis* EO increased cell wall permeability, causing extracellular nucleotide leakage, further facilitating the interaction of EO compounds with bacterial replication receptors, as shown, and as described by Omelon et al. (2016).

This observation is evident when we analyze the results where the addition of EO in the samples containing the four bacteria showed a

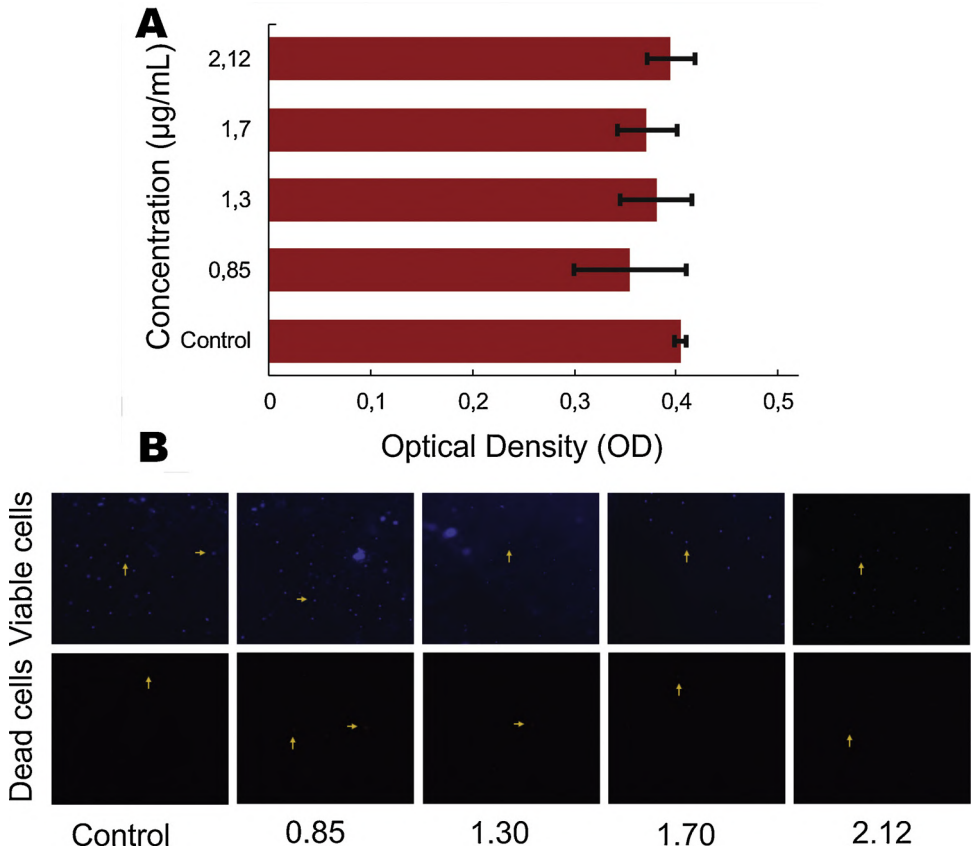


Fig. 4. Cytotoxicity test with human monocytic TPH-1 cells submitted to EO *S. guianensis* at concentrations of 0.87, 1.30, 1.70 and 2.12 µg/mL analyzed by a) absorbances and b) immunofluorescence (Cells analyzed in 10x size with filters to detect blue (viable) and red fluorescence (dead)).

Table 3
Receptors models of four pathogenic bacteria used to analyze the molecular docking with the major constituents of the *Siparuna guianensis* EO.

Strains	Receptor (NCBI database)	Template	Identity (%)	Ramachandran Favored (%)	QMEAN
E. coli ATCC 35218	DNA Polymerase (OTD50424.1)	3K5L	99.06	95.53	−0.42
	RNA Polymerase (KIG77606.1)	2GHY	59.02	97.27	−0.72
P. aeruginosa ATCC 2753	DNA Polymerase (ALZ00656.1)	3E0D	32.39	91.28	−3.24
	RNA Polymerase (OTF50428.1)	5UAQ	67.63	91.64	−2.23
S. aureus ATCC 25923	DNA Polymerase (BBA24323.1)	2XY7	54.01	96.25	−1.54
	RNA Polymerase (OAQ46528.1)	2GHY	57.38	96.36	−0.55
S. Pyogenes ATCC 19615	DNA Polymerase[a]	—	—	—	
	RNA Polymerase (WP_044557961.1)	4JKR	66.36	92.27	−1.95

[a] No model with sufficient identity for homology modeling was found.

higher absorbance ranging from 0.4 to 0.5 and with the RSS antibiotic these values did not exceed 0.4, thus showing the capacity of the *S. guianensis* EO in increase cell permeability.

Studies have been proposed to determine the cytotoxicity of essential oil compounds in human cells, as well as their use as antibacterial agents, including the essential oil of *Minthostachys verticilata* (perperina) (Escobar et al., 2012). We found that the essential oil of *S. guianensis* did not induce cytotoxic effects, as indicated by the MTT assay in TPH1 cells, as well as by immunofluorescence, suggesting that *S. guianensis* essential oil could be safely used as a therapeutic agent.

Florão (2006) reported that the essential oils of *Baccaris dracunculifolia*, *B. cirspa*, *B. gaudichaudiana* and *B. articulata* presented high cytotoxicity at 10 µL/mL for mononuclear cells and granulocytes. In the present study, the concentrations used were not toxic to TPH-1 cells when compared to controls. The concentrations of essential oil that showed better inhibitory effects for *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes*, showed no toxicity in TPH-1 cells.

For a better understanding of the mechanism of interaction between *S. guianensis* EO and bacteria, possible receptors were selected based on the hypotheses formed in the present study, specifically that the bacteria treated with the EO did not replicate. Hu et al. (2019) found that *Litsea cubeba* EO influenced bacterial DNA and RNA replication in methicillin-resistant *Staphylococcus aureus*. Fang et al. (2016) showed through molecular docking that some EO flavonoids can interact with *E.*

Table 4
Molecular Docking results for complexes between major compounds of *Siparuna guianensis* EO and receptors of four pathogenic bacteria.

Bacterial	*E. coli*		*P. aeruginosa*		*S. aureus*		*S. pyogenes*
Receptor	DNA Polymerase	RNA Polymerase	DNA Polymerase	RNA Polymerase	DNA Polymerase	RNA Polymerase	RNA Polymerase
	kcal/mol[a]						
β-Myrcene	−4.0	−2.6	−4.1	−3.7	−4.8	−2.9	−3.7
2-Undecanone	−4.0	−2.7	−3.1	−3.6	−4.4	−3.0	−3.5
γ-Elemene	−5.9	−4.0	−6.6	−5.4	−5.7	−4.4	−5.7
Elixene	−5.9	−4.0	−5.1	−6.2	−5.5	−4.3	−5.6
γ-Muurolene	−6.4	−4.4	−5.8	−6.9	−6.0	−4.1	−5.7
δ-Cadinene	−7.5	−5.2	−6.1	−6.6	−6.3	−4.8	−5.6
Germacrene D	−6.8	−4.9	−5.7	−5.8	−6.2	−4.6	−6.4
Curzerene	−5.7	−3.8	−6.6	−5.7	−6.4	−4.6	−6.1
Spathulenol	−6.7	−5.1	−6.6	−6.9	−6.8	−4.8	−6.2
α-Cadinol	−7.1	−4.5	−5.9	−6.7	−6.4	−4.6	−6.6
Germacrene B	−8.2	−5.3	−7.1	−7.2	−7.1	−5.7	−6.5
Epicurzerenone	−6.3	−4.5	−5.6	−5.8	−6.0	−4.4	−5.8
Rifamycin SV Sodium	−10.9	−6.5	−8.1	−8.4	−8.3	−5.8	−7.5

[a] AutoDockVina affinity energy.

coli DNA.

The *S. guianensis* EO compounds that interact with the active site regions promoted by docking with bacterial DNA and RNA polymerases promote a change in conformation, thereby inhibiting replication. DNA replication, transcription and translation operate with impressive speed and fidelity in bacterial cells; however, these properties are influenced by the action of polymerases (Robinson and Van Oijen, 2013). A change in the conformation of these enzymes can cause great variations in reaction rates, and may even inhibit bacterial replication.

In all living organisms, genomes are replicated by DNA polymerase expressed from the genomes themselves via transcription and translation. This process may undergo changes as a function of external interferences mainly in bacteria (Fujiwara et al., 2013). The DNA and RNA polymerase structures of the bacteria were then modeled as receptors of *S. guianensis* EO ligands. Almost all of them presented identity superior to 30% as indicated by the literature (Xiang, 2006). It was not possible to model DNA polymerase of *S. pyogenes*, which did not have satisfactory identity for homology modeling. The models had Ramachandran favored results above 90% (Giacoppo et al., 2016).

In the present study, we found that the selected molecular targets of *E. coli*, *S. aureus* and *P. aeruginosa* had better values for energy affinity for germacrene B, and only *S. pyogenes* presented better values for α-cadinol, with values very close to those of germacrene B, according to values described in the literature (Shityakov and Förster, 2014). Other authors also reported the bactericidal activity of several EOs containing germacrene B (Oyedeji and Afolayan, 2005; Runyoro et al., 2010; Andrade et al., 2013), suggesting that this molecule has bactericidal action, also considering its interaction in the same region of the

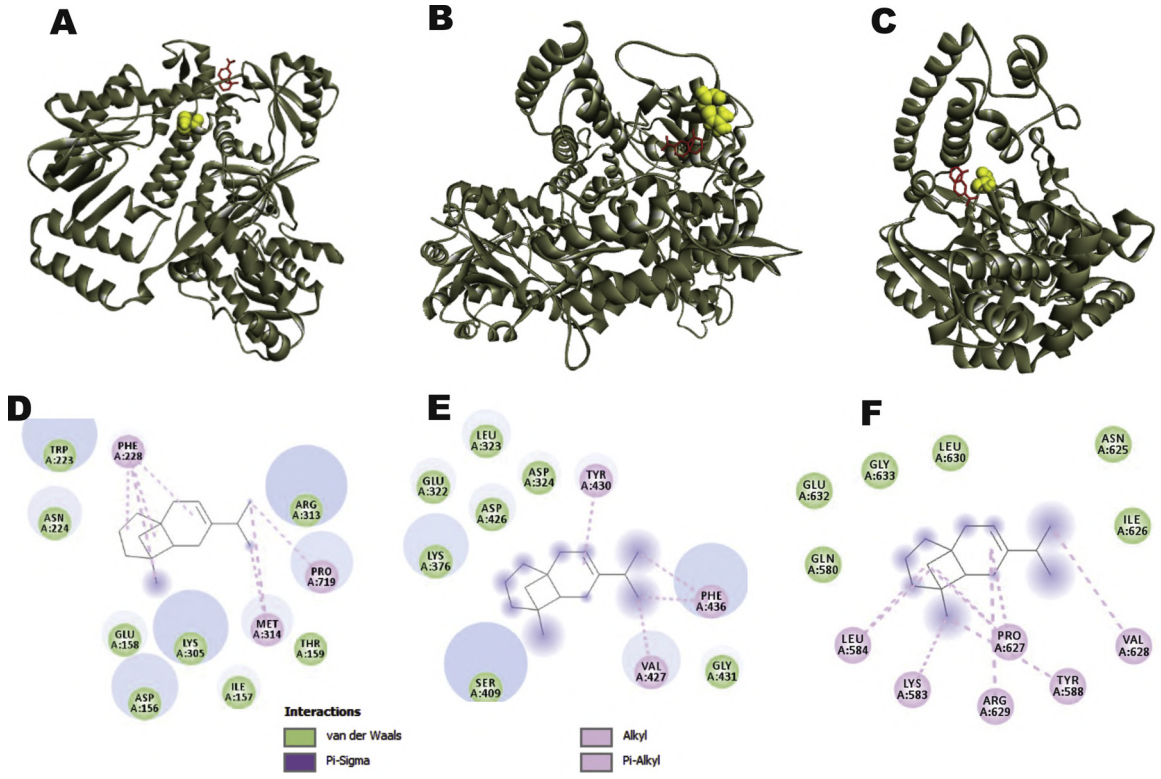


Fig. 5. Germacrene B (Red) complexed with DNA Polymerase enzyme (A, B, C) and 2D maps of molecular interactions with amino acids in DNA Polymerase Active site (Yellow) (D, E, F) of *E. coli* (A, D), *P. aeruginosa* (B, E), *S. aureus* (C, F) and of *E. coli* (D), *P. aeruginosa* (E) and *S. aureus* (F).

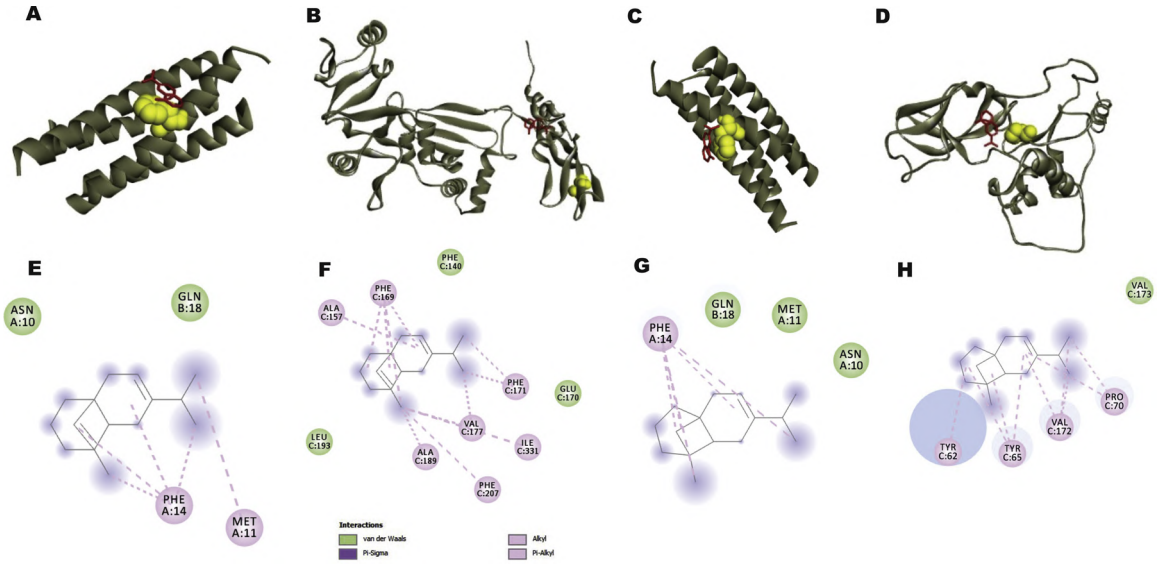


Fig. 6. Germacrene B (Red) complexed with RNA Polymerase enzyme (A, B, C, D) and 2D maps of molecular interactions with amino acids in RNA Polymerase Active site (Yellow) (E, F, G, H) of *E. coli* (A, E), *P. aeruginosa* (B, F), *S. aureus* (C, G) and *S.pyogenes* (D, H).

antibiotic RSS (Ganapathy et al., 2019).

Essential oils have been widely used in various alternative treatments. They inhibit the growth of a wide range of pathogens, mediated by natural compounds produced by plant organs (Swamy et al., 2016). iI is important to note that the unique aroma and other bioactive properties of EOs depend on their chemical constituents.

5. Conclusion

There is increased interest in the use of EOs for control of pathogenic bacteria, such as *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes*. However, quantitative and qualitative variations in the chemical composition is still a complicating factor against the use of EOs that have bactericidal effects. Therefore, it is extremely important to identify the main compounds present in these Eos as well as to understand their mechanisms of action and interactions with vital processes of these organisms in order to better explore their bactericidal activity. S. guianensis EO showed good bactericidal activity, increasing the cell wall permeability of bacteria, and molecular docking analysis showed that germacrene B was a highly reactive molecule when compared to the reference antibiotic. Such knowledge will surely increase the list of products of botanical origin that can be used to reduce the impact of the diseases caused by such pathogenic microorganisms.

CRediT authorship contribution statement

Wellington de Souza Moura: Project administration, Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Silvania Rosa de Souza:** Conceptualization, Methodology. **Fabrício S. Campos:** Formal analysis, Writing - review & editing. **Alex Sander Rodrigues Cangussu:** Resources, Data curation. **Eliane Macedo Sobrinho Santos:** Resources, Data curation. **Bruno Silva Andrade:** Formal analysis, Writing - review & editing. **Cesar Henrique Borges Gomes:** Resources, Data curation. **Kelvinson Fernandes Viana:** Validation, Investigation, Resources, Data curation. **Khalid Haddi:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Eugenio Eduardo Oliveira:** Conceptualization, Validation, Investigation, Formal analysis, Writing - review & editing. **Vitor L. Nascimento:** Validation, Investigation, Resources. **Raimundo Wagner de Souza Aguiar:** Project administration, Supervision, Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2020.112142.

References

Aguiar, R.W.S., Santos, S.F., Morgado, F.S., Ascencio, S.D., Lopes, M.M., Viana, K.F., Didonet, J., Ribeiro, B.M., 2015. Insecticidal and repellent activity of *Siparuna guianensis* Aubl. (negramina) against *Aedes aegypti* and *Culex quinquefasciatus*. PLoS One 10 (2), e0116765. https://doi.org/10.1371/journal.pone.0116765.

Andrade, B.F.M.T., Barbosa, L.N., Probst, I.S., Júnior, A.F., 2013. Antimicrobial activity of essential oils. J. Essent. Oil Res. 26 (1), 34–40.

Arunachalam, K., Ascêncio, S.D., Soares, I.M., Souza Aguiar, R.W., da Silva, L.I., de Oliveira, R.G., de Oliveira Martins, D.T., 2016. *Gallesia integrifolia* (Spreng.) harms: in vitro and in vivo antibacterial activities and mode of action. J. Ethnopharmacol. 184, 128–137. https://doi.org/10.1016/j.jep.2016.03.005.

Bachir, R.G., Benali, M., 2012. Antibacterial activity of the essential oils from the leaves of *Eucalyptus globulus* against *Escherichia coli* and *Staphylococcus aureus*. Asian Pac. J. Trop. Biomed. 2 (9), 739–742. https://doi.org/10.1016/S2221-1691(12)60220-2.

Basera, P., Lavania, M., Agnihotri, A., Lal, B., 2019. Analytical investigation of *Cymbopogon citratus* and exploiting the potential of developed silver nanoparticle against the dominating species of pathogenic bacteria. Front. Microbiol. 10, 282. https://doi.org/10.3389/fmicb.2019.00282.

Bajpai, V.K., Sharma, A., Baek, K.-H., 2013. Antibacterial mode of action of *Cudrania tricuspidata* fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens. Food Control 32 (2), 582–590. https://doi.org/10.1016/j.foodcont.2013.01.032.

Benkert, P., Biasini, M., Schwede, T., 2011. Toward the estimation of the absolute quality of individual protein structure models. Bioinform. 27, 343–350.

Bezic, N., Skocibusic, M., Dinkic, V., Radonic, A., 2003. Composition and antimicrobial activity of Achillea clavennae L. Essential oil. Phytother. Res. 17, 1037–1040.

Borges, J.C.M., Haddi, K., Oliveira, E.E., Andrade, B.S., Nascimento, V.L., Melo, T.S., Didonet, J., Carvalho, J.C.T., Cangussu, A.S., Soares, I.M., Ascencio, S.D., Raposo, N.R.D., Aguiar, R.W.S., 2019. Mosquiticidal and repellent potential of formulations containing wood residue extracts of a Neotropical plant, *Tabebuia heptaphylla*. Ind. Crops Prod. 129, 424–433. https://doi.org/10.1016/j.indcrop.2018.12.022.

Boudjema, K., Guerdouba, A., Hali, L., 2018. Composition, physicochemical analysis, antimicrobial and anti- inflammatory activities of the essential oils obtained from ruta chalepensis l growing wild in northen of Algeria. J. Chem. Soc. Pak. 40 (6), 1054–1062.

Clinical and laboratory standards institute/NCCLS, 2005. Performance Standards for Antimicrobial Susceptibility Testing; Fifteenth Informational Supplement. CLSI/NCCLS Document M100-S15 [ISBN 1-56238-556-9]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.

Cui, H., Zhang, C., Li, C., Lin, L., 2018. Antimicrobial mechanism of clove oil on *Listeria monocytogenes*. Food Control 94, 140–146. https://doi.org/10.1016/j.foodcont.2018.07.007.

Cui, H., Zhang, C., Li, C., Lin, L., 2019. Antibacterial mechanism of oregano essential oil. Ind. Crops Prod. 139, 111498. https://doi.org/10.1016/j.indcrop.2019.111498.
Daly, J.S., Giehl, T.J., Brown, N.C., Zhi, C., Wright, G.E., Ellison, R.T., 2000. In vitro antimicrobial activities of novel anilinouracils which selectively inhibit DNA polymerase III of gram-positive bacteria. Antimicrob. Agents Chemother. 44 (8), 2217–2221.
Dassault Systemes BIOVIA, 2017. Discovery Studio Modeling Environment, Release 2017. Dassault Systemes, San Diego.
El-Banna, T., Abd El-Aziz, A., Sonbol, F., El-Ekhnawy, E., 2019. Adaptation of *Pseudomonas aeruginosa* clinical isolates to benzalkonium chloride retards its growth and enhances biofilm production. Mol. Biol. Rep. 46 (3), 344–3437. https://doi.org/10.1007/s11033-019-04806-7.
Elshafie, H.S., Gruľová, D., Baranová, B., Caputo, L., De Martino, L., Sedlák, V., Camele, I., De Feo, V., 2019. Antimicrobial activity and chemical composition of essential oil extracted from *Solidago canadensis* L. Growing wild in Slovakia. Molecules. 24, 206. https://doi.org/10.3390/molecules24071206.
Escobar, S., Espigares puerto, F., Felip, A., Zanuy, S., Gueguen, M.M., Carrillo, M., Kah, O., Servili, A., 2012. Kisspeptin/GPR54 expression in the brain of the European sea bass. In: Abstract Book of the 7th International Symposium of Fisch Endocrinilogy. Buenos Aires, Argentina. 152.
Fang, Y., Lu, Y., Zang, X., Wu, T., Qi, X., Pan, S., Xu, X., 2016. 3D-QSAR and docking studies of flavonoids as potent *Escherichia coli* inhibitors. Sci. Rep. Ist. Super. Sanita 6 (1). https://doi.org/10.1038/srep23634.
Ferreira, T.P., Kuckelhaus, I.G.P., Tschoeke, P.H., Cangussu, A.S., Borges, J.C.M., Moura, W.S., Aguiar, R.W.S., 2017a. Influence of seasonality on the yield and composition of the essential oil of *Siparuna guianensis* Aublet. African J. Biotechn. 16 (29), 1611–1618. https://doi.org/10.5897/ajb2017.16109.
Ferreira, T.P., Oliveira, E.E., Tschoeke, P.H., Pinheiro, R.G., Maia, A.M.S., Aguiar, R.W., 2017b. Potential use of Negramina (*Siparuna guianensis* Aubl.) essential oil to control wax months and its selectivity in relation to honey bees. Ind. Crops Prod. 109, 151–157. https://doi.org/10.1016/j.indcrop.2017.08.023.
Ferreira, T.P., Haddi, K.F.T., Corrêa, R., Zapata, V.L.B., Piau, T.B., Souza, L.F.N., Aguiar, R.W.S., 2019. Prolonged mosquitocidal activity of *Siparuna guianensis* essential oil encapsulated in chitosan nanoparticles. PLoS Negl. Trop. Dis. 13 (8), e0007624. https://doi.org/10.1371/journal.pntd.0007624.
Florão, A., 2006. Avaliação De Atividades Biológicas De Óleos Essenciais De Quatro Espécies De *Baccharis arteraceae*. Dissertação de mestrado. Programa de Pós-Graduação em Ciencias Farmaceuticas- Universidade Federal do Paraná, Curitiba.
Fujiwara, K., Katayama, T., Nomura, S.M., 2013. Cooperative working of bacterial chromosome replication proteins generated by a reconstituted protein expression system. Nucleic Acids Res. 41, 7176–7183. https://doi.org/10.1093/nar/gkt489.
Ganapathy, U.S., Dartois, V., Dick, T., 2019. Repositioning rifamycins for Mycobacterium abscessus lung disease. Exp. Opin. Drug. Discov. 14 (9), 867–878. https://doi.org/10.1080/17460441.2019.1629414.
Giacoppo, J., de, O.S., Carregal, J.B., Junior, M.C., Cunha, E.F., da, F., Ramalho, T.C., 2016. Towards the understanding of tetrahydroquinolines action in *Aedes aegypti:* larvicide or adulticide? Mol. Simul. 43 (2), 121–133. https://doi.org/10.1080/08927022.2016.1239823.
Haas, J., Barbato, A., Behringer, D., Studer, G., Roth, S., Bertoni, M., Mostaguir, K., Gumienny, R., Schwede, T., 2018. Continuous automated Model Evaluation (CAMEO) complementing the critical assessment of structure prediction in CASP12. Proteins. 86, 387–398. https://doi.org/10.1002/prot.25431.
Heller, A.A., Spence, D.M., 2019. A rapid method for post-antibiotic bacterial susceptibility testing. PLoS One 14 (1), e0210534. https://doi.org/10.1371/journal.pone.0210534.
Hu, W., Li, C., Dai, J., Cui, H., Lin, L., 2019. Antibacterial activity and mechanism of *Litsea cubeba* essential oil against methicillin-resistant *Staphylococcus aureus (MRSA)*. Ind. Crops Prod. 130, 34–41. https://doi.org/10.1016/j.indcrop.2018.12.078.
Kalumbi, M.H., 2019. Effect of Vancomycin, tetracycline, *Persia americana* leaf extract and combinations thereof on antibacterial activity against pathogenic organisms. J. Pharmacogn. Phytochem. 8 (1), 2733–2737.
Kohanski, M.A., Dwyer, D.J., Collins, J.J., 2010. How antibiotics kill bacteria: from targets to networks. Nat. Rev. Microb. 8 (6), 423.
Krishnamoorthy, R., Athinarayanan, J., Periyasamy, V.S., Adisa, A.R., Al-Shuniaber, M.A., Gassem, M.A., Alshatwi, A.A., 2018. Antimicrobial activity of nanoemulsion on drug-resistant bacterial pathogens. Microb. Pathog. 120, 85–96. https://doi.org/10.1016/j.micpath.2018.04.035.
Lennet, E.H. (Ed.), 1985. Manual of Clinical Microbiology, 4th ed. American Society for Microbiology, Washington, DC.
Lee, S.Y., Kim, K.B.W.R., Lim, S.I., Ahn, D.H., 2014. Antibacterial mechanism of *Myagropsis myagroides* extract on *Listeria monocytogenes*. Food Control 42, 23–28. https://doi.org/10.1016/j.foodcont.2014.01.030.
Lin, L., Mao, X., Sun, Y., Cui, H., 2018. Antibacterial mechanism of *artemisinin* / beta-cyclodextrins against methicillin-resistant *Staphylococcus aureus* (MRSA). Microbial Pathogen. 118, 66–73. https://doi.org/10.1016/j.micpath.2018.03.014.
Lourenço, A.M., Haddi, K., Ribeiro, B.M., Corrêia, R.F., Tomé, H.V., Santos-Amaya, O., Pereira, E.J., Guedes, R.N., Santos, G.R., Oliveira, E.E., Aguiar, R.W.S., 2018. Essential oil of *Siparuna guianensis* as an alternative tool for improved lepidopteran control and resistance management practices. Sci. Rep. 8, 7215. https://doi.org/10.1038/s41598-018-25721-0.
Mighri, H., Hajlaoui, H., Akrout, A., Najjaa, H., Neffati, M., 2010. Antimicrobial and antioxidant activities of *Artemisia herba-alba* essential oil cultivated in Tunisian arid zone. C. R. Chimie. 13, 380–386.
Nazzaro, F., Fratianni, F., Martino, L., Coppola, R., Feo, V., 2013. Effect of essential oils on pathogenic bacteria. Pharmaceuticals 6, 1451–1474. https://doi.org/10.3390/ph6121451.
Nocchetti, M., Donnadio, A., Ambrogi, V., Andreani, P., Bastianini, M., Pietrella, D., Latterini, L., 2013. Ag/agcl nanoparticle decorated layered double hydroxides: synthesis, characterization and antimicrobial properties. J. Mater. Chem. B Mater. Biol. Med. 1, 2383–2393. https://doi.org/10.1039/C3TB00561E.
Oliveira, M.S., Cruz, J.N., Mitre, G.P., Costa, W.A., Kataoka, M.S.S., Silva, S.G., Alves, A.C.B.A., Pinheiro, J.J.V., Silva, S.E.M., Menezes, S.A.F., Menezes, T.O.A., Neto, A.M.J.C., Junior, R.N.C., 2019. Antimicrobial, cytotoxic activity of the *Syzygium aromaticum* essential oil, molecular docking and dynamics molecular studies of its major chemical constituent. J. Comp. Theor. Nanosci. 16 (2), 355–364. https://doi.org/10.1166/jctn.2019.8108.
Oliveira, D.M., Melo, F.G., Balogun, S.O., Flach, A., Costa, L.A.M.A., Soares, I.M., Silva, L.I., Ascêncio, S.D., Oliveira Martins, D.T., 2015. Antibacterial mode of action of the hydroethanolic extract of *Leonotis nepetifolia* (L.) R.BR. Involves bacterial membrane perturbations. J. Ethnopharmacol. 172, 356–363.
Omelon, S., Georgiou, J., Habraken, W., 2016. A cautionary (spectral) tail: red-shifted fluorescence by dapi-dapi interactions. Biochem. Soc. Trans. 44, 46–49.
OriginLab, 2007. OriginPro 8, Version 8.0724. OriginLab Corporation, Northampton, MA, USA.
Ostrosky, Elissa A., et al., 2008. Métodos para avaliação da atividade antimicrobiana e determinação da Concentração Mínima Inibitória (CMI) de plantas medicinais. Rev. Bras. Farmacogn. 18 (2), 301–307. https://doi.org/10.1590/S0102-695X2008000200026.
Oyedeji, O.A., Afolayan, A.J., 2005. Chemical composition and antibacterial activity of the essential oil of *Centella asiatica*. Growing in South Africa. Pharm. Biol. 43 (3), 249–252. https://doi.org/10.1080/13880200590928843.
Peyret, M., Carret, G., Carre, C., Fardel, G., Flandrois, J.P., 1990. Mathematical study of the sensitivity curves of *Escherichia coli* exposed to polymyxins. Pathol. Biol. 38, 441–445.
Probst, I.S., 2012. Atividade Antibacteriana De Óleos Essenciais E Avaliação De Potencial Sinérgico. 112f. Dissertação (mestrado) – Universidade Estadual Paulista, Instituto de Biociências de Botucatu.
Rahman, A.A.A., Mohamed, S.M., Samy, E.M., Sayed, M.A., Youssef, E.M.K., Hetta, H.F., 2018. Novel formulation and clinical evaluation of Levofloxacin Hemihydrategel for the treatment of impetigo. J. Am. Sci. 14 (10), 47–57. https://doi.org/10.7537/marsjas141018.06.
Ramachandran, G.N., Sasisekharan, V., 1968. Conformation of polypeptides and proteins. Adv. Protein Chem. 23, 283–437.
Renner, S.S., Hausner, G., 2005. Monograph of Siparunaceae. Flora Neotropica 95, 1–256.
Robinson, A., van Oijen, A.M., 2013. Bacterial replication, transcription and translation: mechanistic insights from single-molecule biochemical studies. Nat. Rev. Microb. 11 (5), 303–315. https://doi.org/10.1038/nrmicro2994.
Roosild, T.P., Castronovo, S., Healy, J., Miller, S., Pliotas, C., Rasmussen, T., Bartlett, Conway, S.J., Booth, I.R., 2010. Mechanism of ligand-gated potassium efflux in bacterial pathogens. Proc. Natl. Acad. Sci. 107, 19784–19789.
Runyoro, D., Ngassapa, O., Vagionas, K., Aligiannis, N., Graikou, K., Chinou, I., 2010. Chemical composition and antimicrobial activity of the essential oils of four *Ocimum* species growing in Tanzania. Food Chem. 119 (1), 311–316. https://doi.org/10.1016/j.foodchem.2009.06.028.
Sanner, M.F., 1999. Python: a Programming Language for Software Integration and Development.
Sanyal, G., Doig, P., 2012. Bacterial DNA replication enzymes as targets for antibacterial drug discovery. Expert Opin. Drug Discov. 7 (4), 327–339.
Sbayou, H., Ababou, B., Boukachabine, K., Manresa, A., Zerouali, K., Amghar, S., 2014. Chemical composition and antibacterial activity of *Artemisia herba-alba* and *Mentha pulegium* essential oils. J. Life Sci. 8, 35–41.
Schrodinger, L.L.C., 2018. The PyMOL Molecular Graphics System, Version 2.0.
Sheoran, P., Tiwari, S.K., 2019. Enterocin LD3 from Enterococcus hirae LD3 causing efflux of intracellular ions and UV-absorbing materials in Gram-negative bactéria. J. Appl. Microb. 126, 1059–1069. https://doi.org/10.1111/jam.14203.
Shityakov, S., Förster, C., 2014. In silico predictive model to determine vector-mediated transport properties for the blood-brain barrier choline transporter. Adv. Appl. Bioinform. Chem. 7, 23–36. https://doi.org/10.2147/AABC.S63749.
Skocibusic, M., Bezić, N., Dunkić, V., Radonić, A., 2004. Antibacterial activity of Achillea clavennae essential oil against respiratory tract pathogens. Fitoterapia 75 (7-8), 733–736. https://doi.org/10.1016/j.fitote.2004.05.009.
Swamy, M.K., Akhtar, M.S., Sinniah, U.R., 2016. Antimicrobial properties of plant essential oils against human pathogens and their mode of action: an updated review. Evid. Complement. Alternat. Med. 3012462. https://doi.org/10.1155/2016/3012462.
Tao, C., Wang, Y., Zhang, X., Li, L., Wu, Y., Han, X., Jiang, X., Lv, Z., 2019. Mechanism of Action of Essential Oils extracted from bamboo (*Phyllostachys heterocycla* cv. *pubescens*) leaves: chemical composition and antimicrobial activity against four food-related microorganisms. Bioresour. 14 (1), 1419–1434.
Trott, O., Olson, A.J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. J. Comput.Chem. 31, 455–461. https://doi.org/10.1002/jcc.21334.
Valentini, C.M.A., Rodriguez-ortiz, C.E., Coelho, M.F.B., 2010. *Siparuna guianensis* aublet (negramina): uma Revisão. Rev. Bras. Plantas Med. 12, 96–104.
Wang, Y., Zhang, Q., Jin, Y., 2019. Epidemiology and antimicrobial susceptibility profiles of extended-spectrum beta-lactamase–producing Klebsiella pneumoniae and Escherichia coli in China. Braz. J. Microbiol. 3, 669–675. https://doi.org/10.1007/s42770-019-00081-7.
Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T., 2018. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 46 (W1), W296–W303.
Xiang, Z., 2006. Advances in homology protein structure modeling. Curr. Protein Pept. Sci. 7 (3), 217–227.
Xu, C., Li, G.-y, Zhang, H., Zu, Y.-n, Li, Z.-h, J-l, Chen, 2018. Antibacterial activity of *Artemisia asiatica* essential oil against some common respiratory infection causing bacterial strains and its mechanism of action in *Haemophilus influenzae*. Microbial. Pathogen. https://doi.org/10.1016/j.micpath.2017.12.032.

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Cassava starch-based essential oil microparticles preparations: Functionalities in mosquito control and selectivity against non-target organisms

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ABSTRACT

Due to the production facilities and great functionalities, the starch extracted from Cassava plants' (*Manihot esculenta* Crantz) roots is one of the most abundant and inexpensive raw materials used in food- and non-food industries. The utilization of starches to encapsulate plant essential oils is a relevant advance in the control of insect pests, including mosquitoes that transmit human diseases. The starch-based microencapsulation of essential oils reduces the degradation and volatilization of active components, providing more sustainable and environmentally friendly activities. Here, we investigated the potential of cassava-based starch microparticle preparations containing the essential oil of a Neotropical plant (*Siparuna guianensis* Aublet) to control larvae of *Aedes aegypti* and *Culex quinquefasciatus*. Moreover, the selectivity of the most efficient microparticles preparation was evaluated on zebrafish embryos (*Danio rerio*), an aquatic non-target organism. The characterization of encapsulated microparticles was achieved by using scanning electron microscopy (SEM), infrared spectroscopy with Fourier transform (FTIR), and thermogravimetric analysis (TGA). Our results revealed an encapsulation efficiency of 82.8 % to 95.3 %, with an average particle diameter of 8.56 μm. Cassava starch microencapsulation reduced the essential oil degradation and enhanced (up to 8 days) the persistent lethal activities (over 50 %) against both species' mosquito larvae compared to the pure essential oil. Furthermore, the exposure of aquatic non-target organisms (embryos of *D. rerio*) revealed these microparticles' adequate selectivity. Collectively, our findings demonstrate that cassava starch-based microparticles exhibit promising functionality as carriers for essential oils with mosquitocidal activities.

1. Introduction

Cassava, *Manihot esculenta* Crantz, is one of the most important food crops in the world, with a production of over 290 million tons in 2020 (Cuenca et al., 2020; Tappiban et al., 2020). With roots containing high starch, cassava is widely used both in human and animal food as well as for non-food purposes in various technological and industrial products (Liu et al., 2019). Its importance in the food industry and its worldwide distribution make the cassava plant a good starch source for encapsulation purposes (Tappiban et al., 2019).

The encapsulation technique has been studied and used to improve some materials' performance through the protection and controlled release of active ingredients (Zaitoon et al., 2021; Annunziata et al., 2020; Alarcón-Alarcón et al., 2018; Tomazelli et al., 2018). Among the polymers of interest, plant starch is an abundant and cheap natural raw material with promising potential for microencapsulation

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(Estevez-Areco et al., 2020; Moura and Ascheri, 2018). In addition to its excellent biodegradation capacity, starch can develop porous spherical aggregates with high encapsulation efficiency. Moreover, starch may provide wall material for microcapsule production to replace high-cost encapsulating agents (Ribeiro and Veloso, 2021; Márquez-Gómez et al., 2017).

The application of encapsulation techniques for delivering particles of plant-based essential oils is a significant advance in the control of not only agricultural insect pests but also insects that transmit human diseases (e.g., mosquitoes). There is an increasing need to develop alternatives to control mosquitoes that are not susceptible to conventional insecticides. Plants have been intensively screened for active products (such as extracts and essential oils) that have insecticidal properties capable of replacing conventional insecticides or being integrated into the management of insect pests (Benelli et al., 2021; Haddi et al., 2020; Isman, 2020; Lucia et al., 2020; Rizzo et al., 2020; Ferreira et al., 2019; Borges et al., 2019; Aguiar et al., 2015). However, some essential oil physicochemical properties (e.g., volatility and miscibility in water) can compromise their persistence in the environment, requiring excessive applications (Benelli et al., 2020; Fuentes et al., 2020; Pavela et al., 2020; Mossa et al., 2017; Pavela, 2015; Asbahani et al., 2015). Indeed, high volatility and low water immiscibility reduce the larvicidal virulence of essential oils when they are directly applied in water bodies. Therefore, we aimed to develop novel strategies based on microencapsulation to increase the residual activity of these alternative bio-insecticides (Fernandes et al., 2014).

Among the promising plants for essential oil production, the aromatic and medicinal Neotropical plant *Siparuna guianensis* Aublet has proven potential in the control of mosquito vectors of human diseases (Ferreira et al., 2019; Aguiar et al., 2015), agricultural pests (Toledo et al., 2019; Lourenço et al., 2018; Ferreira et al., 2017a), and the control of undesired bacteria and fungi (Moura et al., 2020; Oliveira et al., 2020). The combined use of products based on *S. guianensis* essential oils and starches should be extensively studied due to their non-toxicity to humans, animals, and the environment that greatly reduces the impact of insecticides. Furthermore, recent investigations (Ferreira et al., 2019; Kumar et al., 2016) on encapsulated particle preparations used to control mosquito larvae have indicated that these novel strategies do not show any detrimental effects on the aquatic non-target predators of mosquito larvae. However, as observed for other plant-based biorational approaches (Haddi et al., 2020), such practices can not be overlooked in their potential unintended ecotoxicological effects.

Considering that zebrafish (*Danio rerio*) is an excellent animal model for detecting safe levels of environmental contaminants (Bailone et al., 2019) and for human and animal health (Capriello et al., 2020; Chen et al., 2012), investigations that evaluate the detrimental or stimulatory sublethal effects of essential oil starch-based encapsulated preparations on these organisms would allow significant advances in the assessment of the potential ecotoxicological risks for such novel insecticidal alternatives. Here, we encapsulated *S. guianensis* essential oil microparticles using cassava starch and verified whether starch-based encapsulation retained for more extended periods the action of *S. guianensis* essential oil against mosquito (i.e., *Aedes aegypti* and *Culex quinquefasciatus*) larvae. Finally, we assessed the prepared *S. guianensis* microparticles' selectivity to the non-target zebrafish embryos as an indicator of potential toxicity to human and animal health.

2. Materials and methods

2.1. Plant material and essential oil extraction

Leaves of *S. guianensis* were collected between February and May 2019 from trees located in the municipality of Gurupi-Brazil (11°43′45″ S, 49°04′07″ W) and transported for essential oil extraction according to the methods described in Ferreira et al. (2017b). The taxonomic identification of the collected specimens was performed by herbarium specialists from the Federal University of Tocantins (Porto Nacional-TO, Brazil - 10,496). The present investigation was registered in Brazil – SISGEN under number A7CAD12. We extracted the essential oil from the collected *S. guianensis* leaves using the steam distillation method with a Clevenger apparatus as described in Moura et al. (2020). Briefly, we crushed 300 g of fresh *S. guianensis* leaves and mixed the powder with 1000 mL of distilled water. The mixture was placed in a 2000 mL round-bottom flask coupled to Clevenger apparatus. The round-bottom flask mixture was heated, and the vapors produced passed through the condensation column with a cooling system. The condensed essential oil was collected, cooled, and stored in the fridge.

2.2. Mosquito and zebrafish populations

The 3rd instar larvae of *A. aegypti* and *C. quinquefasciatus* used in the experiments were obtained from colonies originally established from field-collected insects from regions where no insecticides have been used for the control of mosquitoes (Aguiar et al., 2015) in the state of Tocantins, Brazil (11°40′55.7″ latitude S, 49°04′3.9″ longitude W). The colonies were maintained under controlled laboratory conditions (T°: 26 °C; RH: 70 %) in the Integrated Pest Management (IPM) laboratory of the Universidade Federal do Tocantins (UFT).

The zebrafish (*D. rerio*) embryos used in the toxicological assays were supplied by the Toxicological Genetics laboratory of the Universidade de Brasilia (UnB). The adult fishes were kept in an automated water recirculating system and provided with water filtered through activated carbon and aerated to eliminate chlorine (ZebTec, Tecniplast, Italy). The physical and chemical characteristics of the system were maintained with pH 7.2–7.6, hardness 6.7°dH, temperature 26 ± 1 °C, and conductivity 728 µS. The aquarium room has a photoperiod of 12 h of light and 12 h of darkness. The fish are fed two to three times a day with a commercial food (SERAVipan ©; Tetramin ©) and live brine shrimps (*Artemia salina* nauplii).

2.3. Chemical analysis of the S. guianensis essential oil

The essential oil's chemical composition was assessed and identified three times by gas chromatography (GC-FID) using a Chemito 8510 GC instrument (Chemito Technologies Ltd, Mumbai, India Pvt.). Separation of the major constituents was performed using a capillary column BP-5 (30 × 0.53 mm i.d., 1.0 mm film thickness), and hydrogen was used as the drag gas at a flow rate of 5 mL/min and a pressure of 20 psi. The GC oven temperature was programmed at 70–210 °C with a 2.5 °C/min heating ramp with the injector and detector temperatures (FID) maintained at 230 °C. The GC–MS analysis was performed on a DSQ MS (Thermo Electron Corporation, Waltham, MA, USA) using a BP-5 (30 × 0.25 × 0.25 mm) capillary column. Helium was used as the drag gas at a 1 mL/min flow rate with a 1:20 split. The column's temperature was programmed to range from 65 to 210 °C using a heating ramp at a rate of 3 °C/min. Mass spectra were obtained in the range of 40–650 amu, operating at 70 V, and the source was maintained at 200 °C following Ferreira et al. (2017b).

2.4. S. guianensis essential oil microparticles preparation

The cassava starch (*M. esculenta*) product (Yoki, General Mills Inc. Yoki, São Bernardo Do Campo, SP, Brazil) used in the microencapsulation process was purchased locally in supermarkets. This starch has a carbohydrate content of 85 %, 12 % humidity, and 3 % other constituents (minerals) with a gelatinization temperature between 58 and 70 °C, as described by Vicentini (2003). The microencapsulation consisted of heating 2 g of starch in 30 mL of water to its gelatinization temperature (68 °C). Then polysorbate was added at a ratio of 2 % with dropwise addition of *S. guianensis* essential oil under constant stirring, and finally, the preparation was cooled to room temperature (Collins et al., 2019). The resulting emulsion was lyophilized by freezing at −20 °C

immediately after preparation. After 24 h, the frozen emulsion was dried for longer than 48 h at −45 °C under a pressure of less than 0.120 mbar using a freeze-drier as described by Samakradhamrongthai et al. (2015) with minor adaptations. We varied the proportions of *S. guianensis* essential oil (O) and starch cassava (S) to create the following samples (O:S): OS1:1, OS1:2, OS1:3, and 0:1 as a control (SG) sample containing only gelatinized starch and polysorbate.

2.5. Morphological and thermogravimetric characterization of S. guianensis essential oil microparticles

The morphology and particle size of the dried microparticles were determined by scanning electron microscopy (SEM) (Ferreira et al., 2019). The lyophilized sample was covered with gold for 180 s using an Emitech K550 (Emitech, Kent, UK) and observed on an SEM Zeiss DSM 962 (Carl Zeiss, Oberkochen, Germany), at 15 kV. The number and mean diameter (with standard deviation) of the microparticles were determined for 400 observations using the software ImageJ (National Institutes of Health, NIH, Maryland, USA). The characterization of microparticles by Fourier-transform infrared spectroscopy (FTIR) spectra was obtained using an IRAffinity^{-1} FTIR (Shimadzu, Kyoto, Japan) coupled to a HATR MIRacle module with a ZnSe prism (Pike technologies, Madison, USA) using 32 scans at a resolution of 4 cm^{-1} from 4000 to 700 cm^{-1}.

The thermogravimetric analysis (TGA) was performed using a TGA-50 instrument (Shimadzu, Kyoto, Japan). The samples were heated at a constant rate of 10 °C min^{-1} in the temperature range of 25–900 °C under nitrogen flow. The starch/essential oil percentage was estimated from the fraction of the dTG area related to the second stage of mass loss followed by an interpolation of the results (Eq. 1).

$$\frac{(fOS - fEO)}{Xos} = \frac{fSG - fEO}{100\%} \quad (1)$$

where:

fSG, fEO, and fOS are the mass fractions of the second peak of degradation in SG, essential oils (EO), and OS samples minus the masses of the water peaks in each analysis, respectively. Xos is equal to the estimated starch fraction in the oil microparticle sample.

The encapsulation efficiency (EE) was estimated using Eq. 2.

$$EE = \left(\frac{Mass\ of\ loaded\ EO}{Mass\ of\ initial\ EO}\right) X\ 100 \quad (2)$$

2.6. Toxicity bioassays

Third-instar larvae of *A. aegypti* and *C. quinquefasciatus* were used to assess the lethal activity of the *S. guianensis* essential oil microparticles encapsulated in cassava starch. Twenty-five larvae were added to a container containing 30 cm^3 of essential oil microparticles solution or a control solution. After a 24 h exposure period, larval mortality was assessed, and all larvae (dead and alive) were removed from the solution. At this time, another group of 25 live larvae was placed into the same solution. This operation was repeated every 24 h until no mortality was observed. The tested solutions consisted of starch microparticles containing *S. guianensis* in three proportions (OS1:1, OS1:2, and OS1:3), an SG control (containing only gelatinized starch and polysorbate), and pure *S. guianensis* essential oil. The tests were performed in four different concentrations as proposed by Aguiar et al. (2015): 0.167, 0.500, 0.834, and 1.667 mg of microparticles /cm^3 of solution.

2.7. Selectivity bioassays

To evaluate the selectivity of *S. guianensis* essential oil microparticles against aquatic non-target organisms, we evaluated lethal (embryo mortality) and sublethal (hatching embryos, cardiac edema, altered yolk sac, and behavioral effects) effects of this alternative insecticide on zebrafish embryos. Our toxicological assays were based on the Fish Embryo Toxicity (FET) tests suggested by the Organization for Economic Co-operation and Development (OECD) toxicity assessment protocols (OECD N° 236, 2013). After collecting the embryos from the aquariums, they were washed and immediately distributed into microplates with the test solutions, allowing them to begin the exposure in the initial embryonic stages. The exposure was performed in 96-well microplates with 200 µL of each concentration. The tests were conducted in a climatic chamber with conditions identical to the cultivation room. The test solutions were prepared with water samples with the same physical and chemical characteristics as those used to cultivate the adult zebrafish. We tested the essential oil microparticle concentrations of 0 (i.e., negative control, with only water); 0.100; 0.171; 0.295; 0.507; 0.807; and 1.500 mg/cm^3 (mg of microparticle/cm^3 of water). For each essential oil microparticle concentration, the tests were performed in triplicate, totalizing 60 organisms. The exposure time was 96 h. We only used the microparticle with an OS1:3 ratio as these microparticles exhibited the highest toxicity for mosquito larvae in the larvicidal bioassays (see the results section for more details).

2.8. Statistical analysis

A mortality over time graph for *A. aegypti* and *C. quinquefasciatus* with the different formulations was plotted using nonlinear regression parameters determined by OriginPro® 8 software. The lethal concentrations (LCs) and effect concentration (EC) of the zebrafish data were determined by probit analysis (Finney, 1971) using POLO PLUS statistical software (Leora Software Berkeley, CA, USA). For the analysis of non-target organisms, the statistical package Sigma Plot 12.5 was used. Unidirectional ANOVA was used to detect differences between groups for normally distributed datasets. When the data did not pass the Kolmogorov-Smirnov normality test, the Levene homogeneity test of variance, Kruskal-Wallis, was used. The Dunnett's test (for parametric analysis) or Dunn' test (for non-parametric analysis) were used to detect significant differences between the tested concentrations and the control. In all analyses, a probability value of less than 0.05 was considered statistically significant ($P < 0.05$).

3. Results

3.1. Chemical analysis of S. guianensis essential oil

Chemical characterization revealed 18 different constituents in the extracted *S. guianensis* essential oil, with the major components being the monoterpene β-myrcene (39.16 %), sesquiterpenes epicurzerenone (16.02 %), and β-copaene (9.33 %) (Table 1).

3.2. Characterization of S. guianensis essential oil microparticles encapsulated with starch cassava

Scanning electron microscopy (SEM) analysis showed that the *S. guianensis* essential oil microparticles presented a smoothly rounded and integral appearance without any surface holes (Fig. 1A). The average size of the particles ranged from 0.6 to 24 µm, with an average diameter of 8.56 ± 5 µm (Fig. 1B).

These microparticles presented broadband between 3600 and 3200 cm^{-1}, characteristic of the hydroxyl functional group, and a band at 3080 cm^{-1}, attributed to the stretching vibration of CH from olefins (Fig. 2). Furthermore, while peaks at 2958, 2926, and 2857 cm^{-1} refer to elongation vibrations, peaks at 1447 and 1377 cm^{-1} refer to angular deformations of C—H bonds of aliphatic chains, respectively (Fig. 2). The C=C double bonds' absorption peaks present in the unsaturated fatty acids were observed at 1603 and 990−800 cm^{-1}. The absorption at 1799 cm^{-1} is characteristic of the ester functional group (Fig. 2).

TGA analysis presented the thermal properties of *S. guianensis* essential oil microparticles and revealed that the calculated

Table 1
Chemical composition, concentrations (%) and Kovats index for the *S. guianensis* essential oil.

Compound	Chemical structure	%	RI[a]	RI Lit.[b]
β-Myrcene		39.16	958	988
Pseudolimonene		0.95	1013	1005
2,6-Dimethyl-3,5,7-octatriene-2-ol, Z,Z-		1.14	1090	1087
β-ylangene		1.64	1216	1272
β-copaene		9.33	1216	1220
2-Undecanone		6.25	1251	1294
β-Bourbonene		0.76	1339	1387
Cyclohexanol, 2-methylene-3-(1-methylethenyl)-, acetate, cis-		2.30	1341	1347
β-elemene		1.99	1398	1389
Elixene		5.88	1431	1492
Epi-cubebol		1.59	1498	1493
Isofuranogermacrene		3.91	1532	1510
Spathulenol		2.73	1536	1577
α-Cadinol		0.86	1580	1602
Epicurzerenone		16.02	1611	1605
Germacrene B		3.97	1613	1591
Reynosin		0.72	1741	1752
Isofuranodienone		0.79	1808	1814
		99.99		

[a] Retention index experimental.
[b] Retention index literature (Adams, 2007; NIST, 2018).

encapsulation efficiency was 82.8 %, 84.8 %, and 95.3 % for the particles of OS1:1, OS1:2, and OS1:3, respectively (Fig. 3).

3.3. Bioassays

Bioassays with *S. guianensis* essential oil microparticles and pure *S. guianensis* essential oil were also evaluated. The toxicity to *A. aegypti* and *C. quinquefasciatus* were obtained for each of the concentrations (Fig. 4). The curve adjustment parameters are described in Supplementary Table 1. The SG (control) particles without essential oil presented an insignificant number of deaths (less than 5 %), indicating the

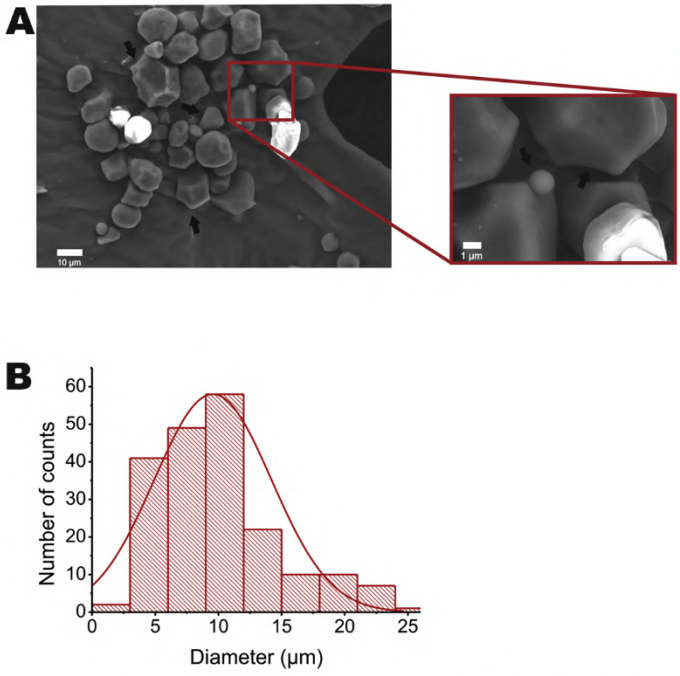

Fig. 1. (**A**) SEM image (with an approximation of 850x) of OS1:1 cassava starch-encapsulated microparticles of *S. guianensis* essential oil. Arrows indicate irregular appearance, forms rounded and integrals, the surface without holes. (**B**) The size distribution of *S. guianensis* essential oil microparticles (n = 400) recorded in SEM images. The mean diameter was 8.56 (± 5, standard deviation) µm.

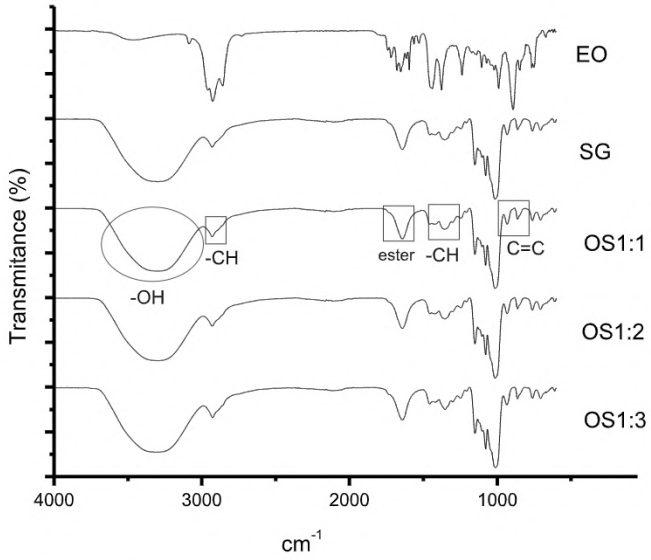

Fig. 2. FTIR spectra of pure *S. guianensis* essential oil and microparticles OS1:1, OS1:2, OS1:3, and SG (control).

absence of any larvicidal activity in this compound. The *S. guianensis* essential oil microparticles OS1:1, OS1:2, and pure *S. guianensis* essential oil did not show high mortality at a concentration of 0.167 mg/cm^3. However, for the OS1:3 microparticles, there was 50 % mortality for approximately three days. This result demonstrates an efficient composition at a low concentration. The OS1:1 microparticles at a concentration of 0.834 mg/cm^3 showed 50 % larvicidal activity for 8.8 and 8.3 days for *A. aegypti* and *C. quinquefasciatus*, respectively. The same mortality was observed for 9.7 days (on *A. aegypti*) and 8.7 days (effect on *C. quinquefasciatus*) when using the OS1:2 microparticles

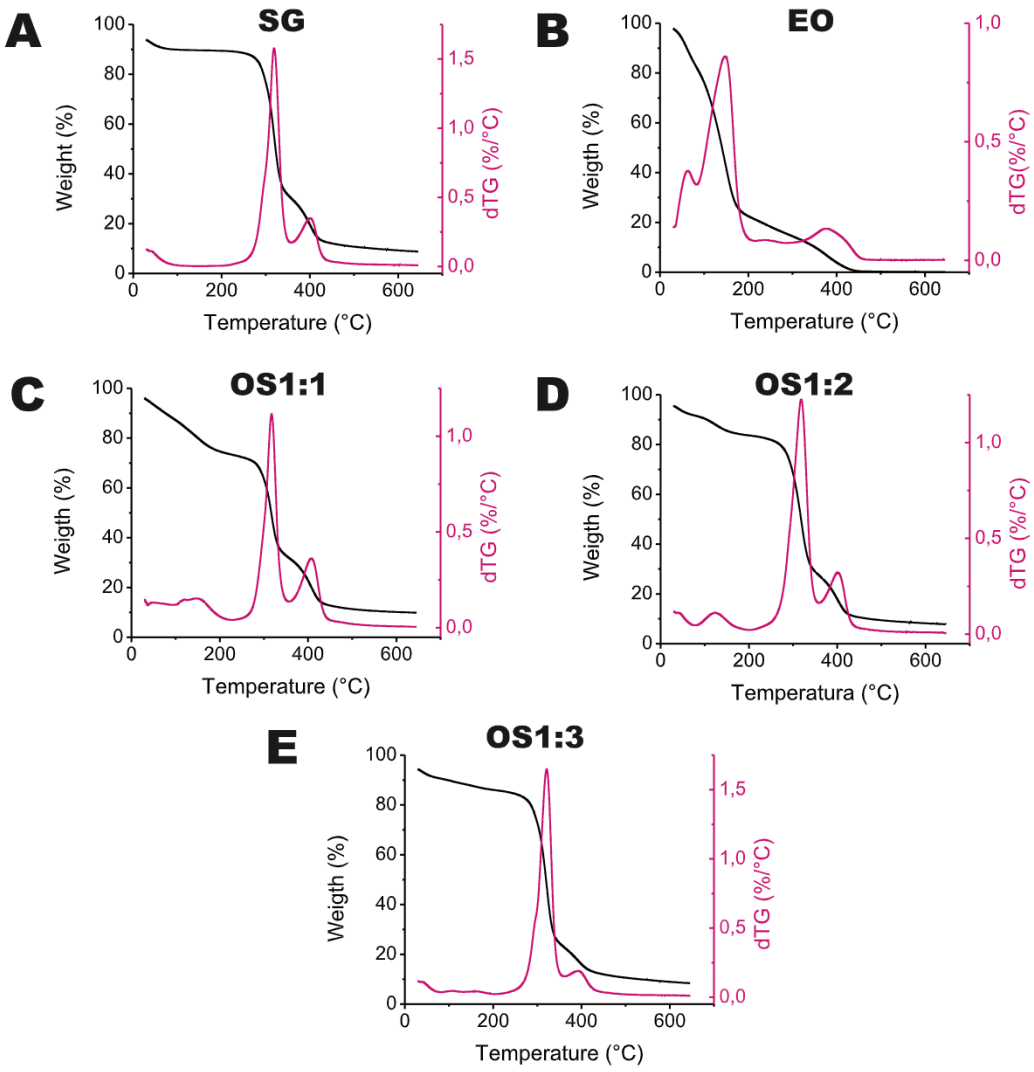

Fig. 3. TGA curves of microparticles. (**A**) SG (control), (**B**) pure *S. guianensis* essential oil, (**C**) OS 1:1, (**D**) OS1:2, (**E**) OS1:3. Black lines represent the residual weight, and magenta lines represent the dTG.

(Fig. 4).

The OS1:3 microparticles were effective at causing a 50 % mortality up to 12.5 days at the same concentration (0.834 mg/cm^3) for controlling *A. aegypti*. In this situation, we observed that the starch fulfills a protective function for *S. guianensis* essential oil, reducing its volatilization, and increasing larvicidal activity. At a concentration of 1.667 mg/cm^3, all microparticles (including pure *S. guianensis* essential oil) caused 100 % mortality on the first test day. This result demonstrates *S. guianensis* essential oil's ability to kill the larvae of *A. aegypti* and *C. quinquefasciatus* (Fig. 4).

3.4. Selectivity non-target organism

The *S. guianensis* essential oil OS1:3 proportion microparticles (i.e., the most toxic against larvae of both *A. aegypti* and *C. quinquefasciatus* mosquitoes) homogeneously diluted in all the concentrations used, and an overview of the embryotoxicity results are shown in Fig. 5. After 24 h of exposure, approximately 20 % of the zebrafish embryos exposed to the highest concentration (1.500 mg/cm^3) had died. At 48 h, only organisms exposed to concentrations of 0.807 and 1.500 mg/cm^3 had died, with death rates of more than 30 % and 100 %, respectively. After 96 h, the embryos exposed to a concentration of 0.507 mg/cm^3 hatched at a rate above 50 %, and the estimated LC_{50} value was 0.936 mg/cm^3 (Fig. 5). The sublethal effects were observed until the last day of exposure (96 h), and it was possible to determine EC_{50} values for altered yolk sac (0.374 mg/cm^3, Fig. 6A) and cardiac edema (0.283 mg/cm^3, Fig. 6B). Furthermore, exposure to essential oil-microencapsulated microparticles sublethally altered the side swimming (for 40 % of embryos exposed to 0.295 mg/cm^3) and back swimming (for 50 % of tested embryos exposed to 0.507 mg/cm^3) of zebrafish embryos (Fig. 7). Behavioral changes were not observed in any of the experiments using lower concentrations.

4. Discussion

We demonstrated cassava starch's potential to encapsulate and carry particles of the essential oil of *S. guianensis*. The use of cassava starch promoted long-lasting activities of this alternative insecticide against larvae of *A. aegypti* and *C. quinquefasciatus*. We then determined the thermal characteristics of the microencapsulates and the microencapsulation efficiency. This research revealed that the cassava starch-based *S. guianensis* microparticles exhibited good selectivity against embryos of zebrafish *D. rerio*. This result proved that this product is safe for use in aquatic environments.

Our chromatographic analysis revealed that the major components in the *S. guianensis* essential oil are β-myrcene (39.16 %), epicurzerenone (16.02 %), and β-copaene (9.33 %). These components have been shown to be toxic to some agricultural and urban pests (Sun et al., 2020;

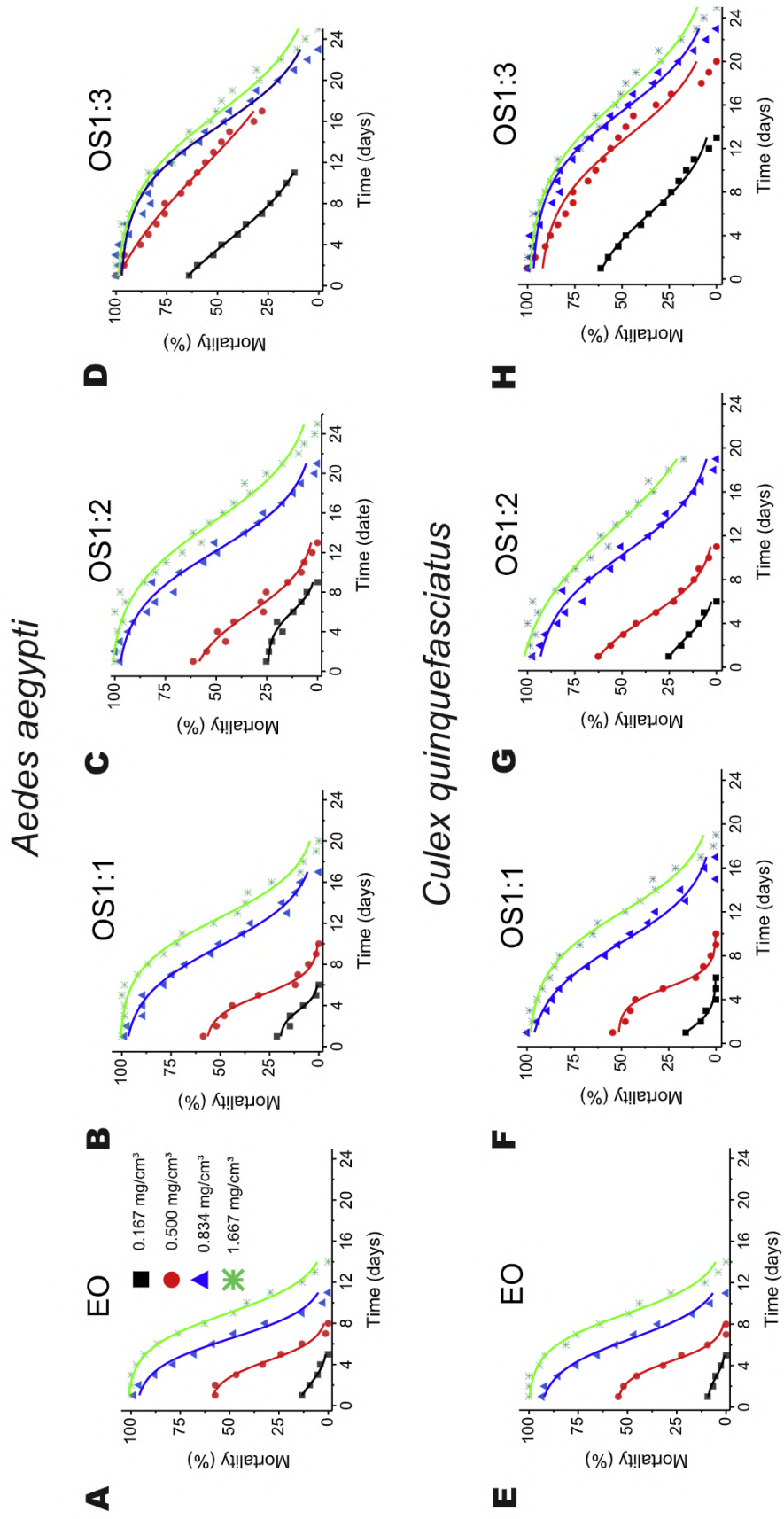

Fig. 4. Residual activities of cassava starch-encapsulated microparticles of *S. guianensis* essential oil (at different concentrations) against larvae of *A. aegypti* and *C. quinquefasciatus* mosquitoes. The parameters of the nonlinear regressions are described in Supplementary Table 1. Each symbol represents the average of three replicates.

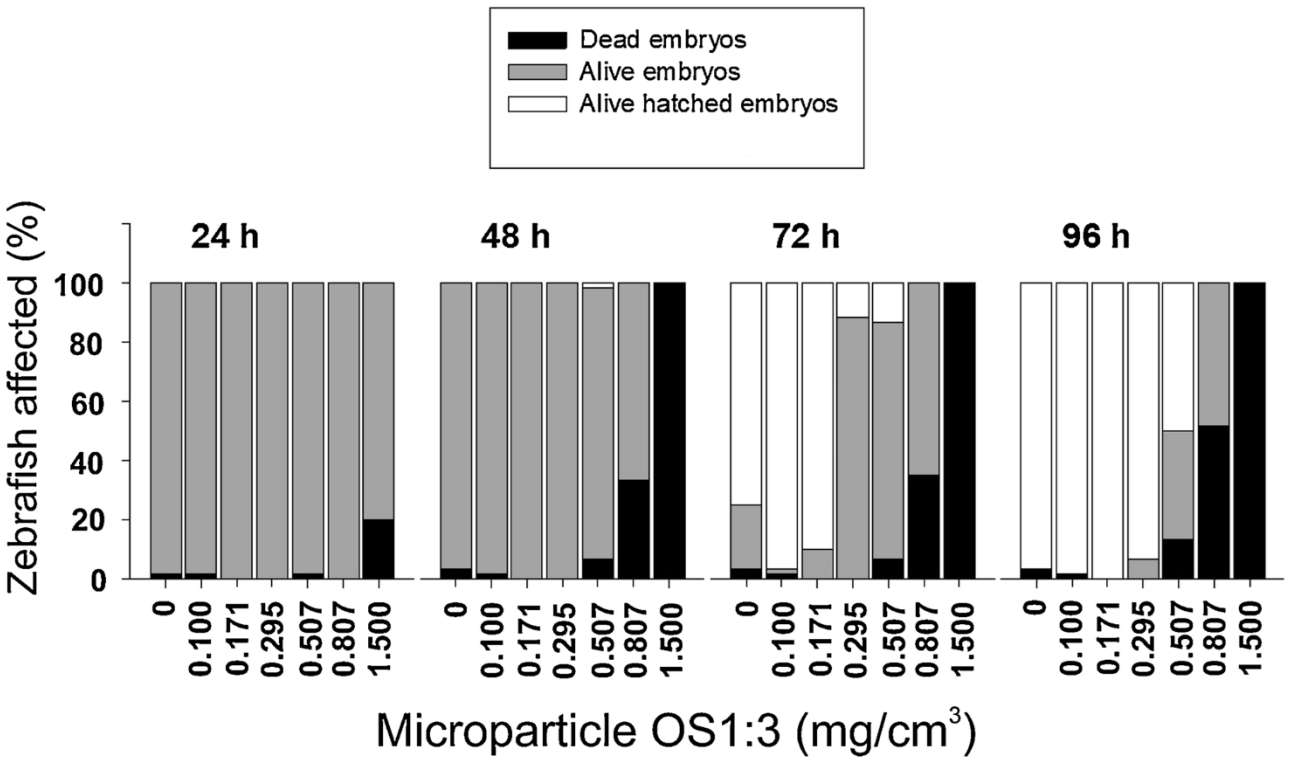

Fig. 5. Toxicity of the OS1:3 *S. guianensis* essential oil microparticle with different concentrations to zebrafish embryos.

Peterson et al., 2020), but their use as insecticides requires some adjustments because they are volatile nonpolar molecules. Therefore, we microencapsulated the *S. guianensis* essential oil with cassava starch to reduce the volatility-related losses of efficiency. The positive result of our technique was a combination of effects, including encapsulation efficiency, microparticles structure, and an interaction between the carrier (i.e., cassava starch) and the different concentrations of essential oil.

The encapsulation efficiency was determined using TGA curves. The encapsulation efficiency ranged from 82.8 % to 95.3 %, which are better results than those reported by Samakradhamrongthai et al. (2015), who obtained 66.74 % efficiency using octenyl succinic anhydride starch. Such differences in efficiency may be associated with the use of polysorbate, as this compound acts as an emulsifying agent, improving the interaction between the essential oil and the starch (Kishore et al., 2011). Therefore, we were able to record the microcapsule degradation in three stages: *1)* the evaporation of water at temperatures up to 110 °C, *2)* further degradation starting at a temperature of 279 °C with the maximum degradation at 376 °C, and *3)* the final degradation starting at 380 °C and ending at 425 °C. The first stage involved the degeneration of water molecules, causing small peaks. The second stage temperature is associated with the interaction between starch and the essential oil, as recorded by Fortunati et al. (2016), and the third stage may represent the elements with long chains and polysorbate described by Kishore et al. (2011).

At the structural level, our prepared microencapsulates were integral, and without holes on their surface, which are good indicators of stability (Murúa-Pagola et al., 2009). Their average size (8.56 μm) was smaller than the size of the microparticles similarly encapsulated with starch reported in previous studies and ranged from 30 to 40 μm (Murúa-Pagola et al., 2009; Márquez-Gómez et al., 2017). Differences in the size of the encapsulated particles are generally influenced by the technique used, where a higher temperature may cause the fusion of two or more microparticles, thereby providing larger diameter microparticles (Glenn et al., 2010).

Furthermore, the ratio of oil to starch can reduce efficiency losses through encapsulation efficiency and the physical structure. An excess of oil could result in a rough surface, influencing microparticles' size (Márquez-Gómez et al., 2017). The OS1:3 sample showed small differences in the FTIR results in relation to the microparticle SG (control). Such differences were due to the lower proportion of essential oil used in its preparation, thus suffering less structure variation. As the proportion of essential oil increased in the microparticles OS1:1 and OS1:2, we observed differences in the microparticles structure. This result is in concordance with previous reports that used starch for essential oil encapsulation (Tongdeesoontorn et al., 2011), increasing the peak intensity at 934 cm^{-1} in relation to the other peaks. The bands occurring in the 1000 cm^{-1} region are related to the starch's crystalline structure, which is related to its retrogradation after the lyophilization process (Vicentini, 2003).

After verifying that the *S. guianensis* essential oil microparticles encapsulated in cassava starch possessed good stability, these preparations were used in toxicological bioassays. Their larvicidal activity was compared to the activity of pure *S. guianensis* essential oil. Our results revealed that the pure essential oil showed greater larvicidal activity in the initial days but significantly decreased over time. This result is probably due to the lower water immiscibility of the pure essential oil and its components' high volatility (Portella et al., 2014). Using an encapsulation technique to protect and slow the essential oil release has been demonstrated in previous investigations (Peres et al., 2020; González et al., 2016; Bringas-Lantigua and Pino, 2012). In our investigation, the starch layer acted as an essential oil protector and contributed to a longer larval mortality period. Our results with the OS1:3 microparticles at a concentration of 1.667 mg/cm^3 showed an increase of more than 80 % in the lethal activity time (over 50 %) compared to pure *S. guianensis* essential oil.

Furthermore, the increase in the starch proportion in the microcapsules, while maintaining the same concentrations of pure *S. guianensis* essential oil, promoted an adequate level of larvicidal activity for longer periods. They effectively killed mosquito larvae for up to 24 days, as observed in the OS1:3 microparticles for both *A. aegypti* and *C. quinquefasciatus* larvae. Moreover, the OS1:3 microparticles at the

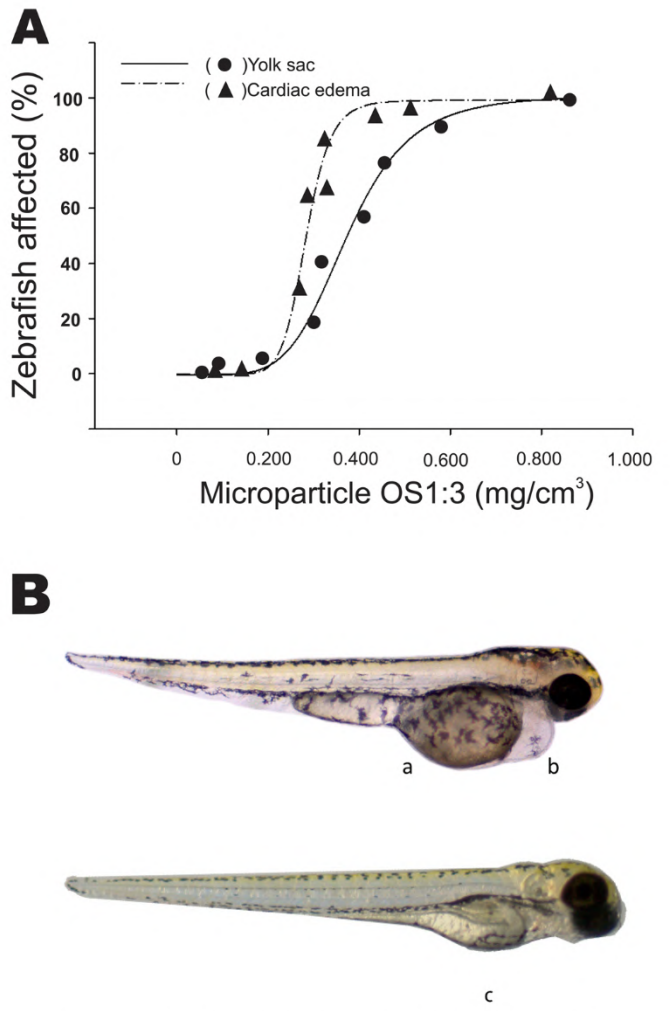

Fig. 6. (**A**) Percentage of zebrafish embryos with alterations on yolk sac (filled circles) or exhibiting cardiac edema (filled triangles) after 96 h of exposure to OS1:3 *S. guianensis* essential oil microparticles. Each symbol represents the average of three replicates. Lines represent the fit obtained by using the probit model for each data set. (**B**) Representation of a zebrafish embryo exhibiting altered yolk sac (a) and cardiac edema (b) comparatively with an embryo without alterations (c).

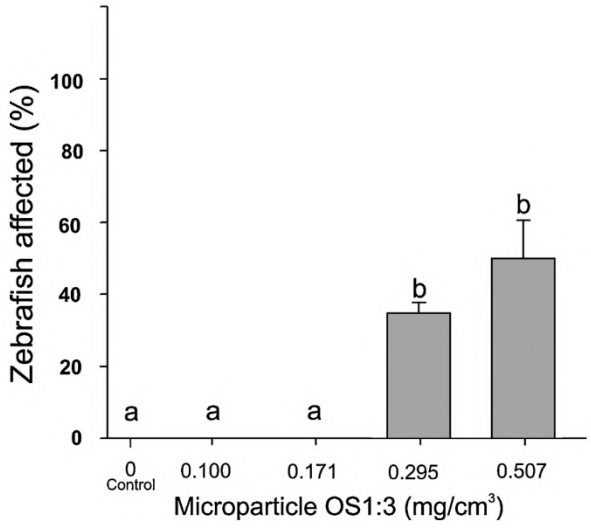

Fig. 7. Percentage of zebrafish embryos exhibiting altered swimming behaviors (side and back swimming) after the exposure (96 h) to OS1:3 *S. guianensis* essential oil microparticles. Data are presented as mean ± standard deviation (SD). Groups with different letters are significantly different ($P < 0.05$) compared to control.

lowest concentration used (0.167 mg/cm^3) killed 50 % of the larvae of *A. aegypti* (at 2.7 days) and *C. quinquefasciatus* (at 2.3 days). Therefore, our formulation performed better and for longer periods than chitosan-based microparticles containing double the active ingredient concentration (0.374 mg/cm^3) of *S. guianensis* essential oil (Ferreira et al., 2019). This result reinforces the great potential of our cassava starch-based microencapsulation technique. Our research supports previous investigations that have reported the efficiency of starch-based microencapsulation of essential oils to control insect microbial pests (Ahsaei et al., 2019; López et al., 2014; Tomazelli et al., 2018).

In addition to the prolongation of larvicidal activity, our results revealed that the *S. guianensis* essential oil microparticles also presented low toxicity to zebrafish embryos. The zebrafish embryos were selected as a non-target aquatic organism showing high genomic homology with human (> 70 %) and similarity of physiological responses, making it an attractive model organism to evaluate food toxicity and use to develop novel medicines (Haddad et al., 2019; Sanjeewa et al., 2018; Kang et al., 2013). Here, after the 96 h exposure period and using the most efficient larvicidal cassava starch-based microparticles (i.e., OS1:3 microparticles), the LC_{50} for the zebrafish embryos was 0.936 mg/cm^3, which is far beyond the toxicity level (i.e., 0.100 mg/cm^3) preconized by the Organization for economic co-operation and development (OECD, 2013). Furthermore, even for sublethal effects (e.g., embryo hatching), the impact of the cassava starch-based *S. guianensis* essential oil microparticles could be observed only at concentrations higher than 0.507 mg/cm^3, which are more than 100-fold higher than those concentrations (0.05 mg/cm^3) recorded for investigations using *Cyperus articulates* essential oil (Brillatz et al., 2020).

5. Conclusion

Cassava starch demonstrated an excellent capacity to encapsulate the *S. guianensis* essential oil and produced microparticles that exhibited prolonged activity against *A. aegypti* and *C. quinquefasciatus* mosquitoes. Furthermore, these cassava starch-based *S. guianensis* essential oil microparticles presented low toxicity to non-target aquatic organisms (embryos of zebrafish). This result reinforces the potential of environmentally friendly alternatives available for mosquito control.

CRediT authorship contribution statement

Wellington S. Moura: Project administration, Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Eugênio E. Oliveira:** Conceptualization, Validation, Investigation, Formal analysis, Writing - review & editing. **Khalid Haddi:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Roberto F.T. Corrêa:** Formal analysis, Methodology. **Tathyana B. Piau:** Resources, Data curation. **Diego S. Moura:** Formal analysis. **Suetônio F. Santos:** Resources, Data curation. **Cesar K. Grisolia:** Validation, Investigation, Formal analysis, Data curation. **Bergmann M. Ribeiro:** Validation, Investigation, Resources, Data curation. **Raimundo Wagner S. Aguiar:** Project administration, Supervision, Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2021.113289.

References

Adams, R., 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, fourth ed. Allured Publishing Corp., Carol Stream, IL, USA.

Aguiar, R.W.S., Santos, S.F., Morgado, F.S., Ascencio, S.D., Lopes, M.M., Viana, K.F., Didonet, J., Ribeiro, B.M., 2015. Insecticidal and repellent activity of *Siparuna guianensis* Aubl. (negramina) against *Aedes aegypti* and *Culex quinquefasciatus*. PLoS One 10 (2), e0116765. https://doi.org/10.1371/journal.pone.0116765.

Ahsaei, S.M., Rodríguez-Rojo, S., Salgado, M., Cocero, M.J., Jahromi, K.T., Amoabediny, G., 2019. Insecticidal activity of spray dried microencapsulated essential oils of *Rosmarinus officinalis* and *Zataria multiflora* against Tribolium confusum. Crop. Prot., 104996 https://doi.org/10.1016/j.cropro.2019.104996.

Alarcón-Alarcón, C., Inostroza-Riquelme, M., Torres-Gallegos, C., Araya, C., Miranda, M., Sánchez-Caamaño, J.C., Moreno-Villoslada, I., Oyarzun-Ampuero, F.A., 2018. Protection of astaxanthin from photodegradation by its inclusion in hierarchically assembled nano and microstructures with potential as food. Food Hydrocoll. 83, 36–44. https://doi.org/10.1016/j.foodhyd.2018.04.033.

Annunziata, G., Jiménez-García, M., Capó, X., Moranta, D., Arnone, A., Tenore, G.C., Sureda, A., Tejada, S., 2020. Microencapsulation as a tool to counteract the typical low bioavailability of polyphenols in the management of diabetes. Food Chem. Toxicol. 139, 111248 https://doi.org/10.1016/j.fct.2020.111248.

Asbahani, A.E., Miladi, K., Badri, W., Sala, M., Addi, E.H.A., Casabianca, H., Mousadik, A.E., Hartmann, D., Jilale, A., Renaud, F.N.R., Elaissari, A., 2015. Essential oils: from extraction to encapsulation. Int. J. Pharm. 483 (1–2), 220–243. https://doi.org/10.1016/j.ijpharm.2014.12.069.

Bailone, R.L., Aguiar, L.K., Roca, R.O., Borra, R.C., Corrêa, T., Janke, H., Silva, H.C., 2019. Zebrafish as an animal model for food safety research: trends in the animal research. Food Biotechnol. 33 (4), 283–302. https://doi.org/10.1080/08905436.2019.1673173.

Benelli, G., Pavela, R., Cianfaglione, K., Sender, J., Danuta, U., Maślanko, W., Canale, A., Barboni, L., Petrelli, R., Zeppa, L., Aguzzi, C., Maggi, F., 2020. Ascaridole-rich essential oil from marsh rosemary (*Ledum palustre*) growing in Poland exerts insecticidal activity on mosquitoes, moths and flies without serious effects on non-target organisms and human cells. Food Chem. Toxicol. 138, 111184 https://doi.org/10.1016/j.fct.2020.111184.

Benelli, G., Rizzo, R., Zeni, V., Govigli, A., Samkova, A., Sinacori, M., Lo Verde, G., Pavela, R., Cappellacci, L., Petrelli, R., Spinozzi, E., Morshedloo, M.R., Maggi, F., Canale, A., 2021. *Carlina acaulis* and *Trachyspermum ammi* essential oils formulated in protein baits are highly toxic and reduce aggressiveness in the medfly, *Ceratitis capitata*. Ind. Crops Prod. 161, 113191 https://doi.org/10.1016/j.indcrop.2020.113191.

Borges, J.C.M., Haddi, K., Oliveira, E.E., Andrade, B.S., Nascimento, V.L., Melo, T.S., Didonet, J., Carvalho, J.C.T., Cangussu, A.S., Soares, I.M., Ascencio, S.D., Raposo, N. R.D., Aguiar, R.W.S., 2019. Mosquiticidal and repellent potential of formulations containing wood residue extracts of a Neotropical plant, *Tabebuia heptaphylla*. Ind. Crops Prod. 129, 424–433. https://doi.org/10.1016/j.indcrop.2018.12.022.

Brillatz, T., Jacmin, M., Queiroz, E.F., Marcourt, L., Slacanin, I., Petit, C., Carrupt, P., Bum, E.N., Herrling, P., Crawford, A.D., Wolfender, J., 2020. Zebrafish bioassay-guided isolation of antiseizure compounds from the Cameroonian medicinal plant *Cyperus articulatus* L. Med. Plants - Int. J. Phytomedicines Relat. Ind. 70, 153175 https://doi.org/10.1016/j.phymed.2020.153175.

Bringas-Lantigua, M., Pino, J.A., 2012. Microencapsulacion de saborizantes mediante secado por atomizacion. Instituto De Investigaciones Para La Industria Alimentaria 11 (2), 34–68.

Capriello, T., Visone, I.M., Motta, C.M., Ferrandino, I., 2020. Adverse effects of E150d on zebrafish development. Food Chem. Toxicol., 111877 https://doi.org/10.1016/j.fct.2020.111877.

Chen, Y., Yang, Z., Wen, C., Chang, Y., Wang, B., Hsiao, C., Shih, T., 2012. Evaluation of the structure–activity relationship of flavonoids as antioxidants and toxicants of zebrafish larvae. Food Chem. 134 (2), 717–724. https://doi.org/10.1016/j.foodchem.2012.02.166.

Collins, S., York, D.W., Kazmi, S., Mohammed, A.K., 2019. Formation of wax walled microcapsules via double emulsion using cross membrane emulsification at elevated temperatures. J. Food Eng., 109739 https://doi.org/10.1016/j.jfoodeng.2019.109739.

Cuenca, P., Ferrero, S., Albani, O., 2020. Preparation and characterization of cassava starch acetate with high substitution degree. Food Hydrocoll. 100, 105430 https://doi.org/10.1016/j.foodhyd.2019.105430.

Estevez-Areco, S., Guz, L., Candal, R., Goyanes, S., 2020. Active bilayer films based on cassava starch incorporating ZnO nanorods and PVA electrospun mats containing rosemary extract. Food Hydrocoll. 108, 106054 https://doi.org/10.1016/j.foodhyd.2020.106054.

Fernandes, R.V.B., Borges, S.V., Botrel, D.A., 2014. Gum arabic / starch / maltodextrin / inulin as wall materials on the microencapsulation of rosemary essential oil. Carbohyd. Polym. 101, 524–532. https://doi.org/10.1016/j.carbpol.2013.09.083.

Ferreira, T.P., Oliveira, E.E., Tschoeke, P.H., Pinheiro, R.G., Maia, A.M.S., Aguiar, R.W. S., 2017a. Potential use of Negramina (*Siparuna guianensis* Aubl.) essential oil to control wax months and its selectivity in relation to honey bees. Ind. Crops Prod. 109, 151–157. https://doi.org/10.1016/j.indcrop.2017.08.023.

Ferreira, T.P., Kuckelhaus, I.G.P., Tschoeke, P.H., Cangussu, A.S., Borges, J.C.M., Moura, W.S., Aguiar, R.W.S., 2017b. Influence of seasonality on the yield and composition of the essential oil of *Siparuna guianensis* Aublet. Afr. J. Biotechnol. 16 (29), 1611–1618. https://doi.org/10.5897/ajb2017.16109.

Ferreira, T.P., Haddi, K., Correa, R.F.T., Zapata, V.L.B., Piau, T.B., Souza, L.F.N., Santos, S.G., Oliveira, E.E., Jumbo, L.O.V., Ribeiro, B.M., Grisolia, C.K., Fidelis, R.R., Aguiar, R.W.S., 2019. Prolonged mosquitocidal activity of *Siparuna guianensis* essential oil encapsulated in chitosan nanoparticles. PLoS Negl. Trop. Dis. 9, 1–23. https://doi.org/10.1371/journal.pntd.0007624.

Finney, D.J., 1971. Probit Analysys, 3th ed. Cambridge University Press, London, p. 25.

Fortunati, E., Gigli, M., Luzi, F., Lotti, N., Munari, A., Gazzano, M., Kenny, J.M., 2016. Poly(Butylene cyclohexanedicarboxylate/diglycolate) random copolymers reinforced with SWCNTs for multifunctional conductive biopolymer composites. Express Polym. Lett. 10 (2), 111–124. https://doi.org/10.3144/expresspolymlett.2016.12.

Fuentes, C., Ruiz-Rico, M., Fuentes, A., Barat, J.M., Ruiz, M.J., 2020. Comparative cytotoxic study of silica materials functionalised with essential oil components in HepG2 cells. Food Chem. Toxicol., 111858 https://doi.org/10.1016/j.fct.2020.111858.

Glenn, G.M., Klamczynski, A.P., Woods, D.F., Chiou, B., Orts, W.J., Imam, S.H., 2010. Encapsulation of plant oils in porous starch microspheres. J. Agric. Food Chem. 58, 4180–4184. https://doi.org/10.1021/jf9037826.

González, A., Martínez, M.L., Paredes, A.J., León, A.E., Ribotta, P.D., 2016. Study of the preparation process and variation of wall components in chia (*Salvia hispanica* L.) oil microencapsulation. Powder Technol. 301 (2016), 868–875. https://doi.org/10.1016/j.powtec.2016.07.02.

Haddad, J.G., Picard, M., Bénard, S., Desvignes, C., Desprès, P., Diotel, N., El Kalamouni, C., 2019. *Ayapana triplinervis* Essential oil and its main component Thymohydroquinone Dimethyl ether inhibit zika virus at doses devoid of toxicity in zebrafish. Molecules 24 (19), 3447. https://doi.org/10.3390/molecules24193447.

Haddi, K., Turchen, L.M., Viteri Jumbo, L.O., Guedes, R.N., Pereira, E.J., Aguiar, R.W.S., Oliveira, E.E., 2020. Rethinking biorational insecticides for pest management: unintended effects and consequences. Pest Manag. Sci. 76, 2286–2293. https://doi.org/10.1002/ps.5837.

Isman, B.M., 2020. Botanical Insecticides in the Twenty-First Century—Fulfilling Their Promise? Annu. Rev. Entomol. 65, 233–249. https://doi.org/10.1146/annurev-ento-011019-025010.

Kang, M., Cha, S.H., Wijesinghe, W.A.J.P., Kang, S., Lee, S., Kim, E., Song, C.B., Jeon, Y., 2013. Protective effect of marine algae phlorotannins against AAPH-induced oxidative stress in zebrafish embryo. Food Chem. 138 (2–3), 950–955. https://doi.org/10.1016/j.foodchem.2012.11.005.

Kishore, R.S., Pappenberger, A., Dauphin, I.B., Ross, A., Buergi, B., Staempfli, A., Mahler, H.C., 2011. Degradation of polysorbates 20 and 80: studies on thermal autoxidation and hydrolysis. J. Pharmac. Sci. 100 (2), 721–731. https://doi.org/10.1002/jps.22290.

Kumar, P.M., Murugan, K., Madhiyazhagan, P., Kovendan, K., Amerasan, D., Chandramohan, B., Dinesh, D., Suresh, U., Nicoletti, M., Alsalhi, M.S.A., Devanesan, S., Wei, H., Kalimuthu, K., Hwang, J., Iacono, A.L., Benelli, G., 2016. Biosynthesis, characterization, and acute toxicity of *Berberis tinctoria*-fabricated silver nanoparticles against the Asian tiger mosquito, *Aedes albopictus*, and the mosquito predators *Toxorhynchites splendens* and *Mesocyclops thermocyclopoides*. Parasitol. Res. 115 (2), 751–759. https://doi.org/10.1007/s00436-015-4799-y.

Liu, R., Sun, W., Zhang, Y., Huang, Z., Hu, H., Zhao, M., 2019. Preparation of starch dough using damaged cassava starch induced by mechanical activation to develop staple foods: application in crackers. Food Chem. 271, 284–290. https://doi.org/10.1016/j.foodchem.2018.07.202.

López, A., Castro, S., Andina, M.J., Ures, X., Munguía, B., Llabot, J.M., Elder, H., Dellacassa, E., Palma, S., Domínguez, L., 2014. Insecticidal activity of microencapsulated *Schinus molle* essential oil. Ind. Crops Prod. 53, 209–216. https://doi.org/10.1016/j.indcrop.2013.12.038.

Lourenço, A.M., Haddi, K., Ribeiro, B.M., Corrêia, R.F., Tomé, H.V., Santos-Amaya, O., Pereira, E.J., Guedes, R.N., Santos, G.R., Oliveira, E.E., Aguiar, R.W.S., 2018. Essential oil of *Siparuna guianensis* as an alternative tool for improved lepidopteran control and resistance management practices. Sci. Rep. 8, 7215. https://doi.org/10.1038/s41598-018-25721-0.

Lucia, A., Girard, C., Fanucce, M., Coviella, C., Rubio, R.G., Ortega, F., Guzmán, E., 2020. Development of an environmentally friendly larvicidal formulation based on essential oil compounds blend to control *Aedes aegypti* larvae: correlations between physico-chemical properties and insecticidal activity. ACS Sus. Chem. Eng. 8 (29), 10995–11006. https://doi.org/10.1021/acssuschemeng.0c03778.

Márquez-Gómez, M., Galicia-García, T., Márquez-Melendez, R., Ruíz-Gutierrez, M., Quintero-Ramos, A., 2017. Spray-dried microencapsulation of orange essential oil

using modified rice starch as wall material. J. Food Process. Preserv. 42 (2), e13428 https://doi.org/10.1111/jfpp.13428.
Mossa, A.T.H., Abdelfattah, N.A.H., Mohafrash, S.M.M., 2017. Nanoemulsion of camphor (*Eucalyptus globules*) essential oil, formulation, characterization and insecticidal activity against wheat weevil, *Sitophilus granaries*. Asian J. of Crop Sci. 9, 50–62. https://doi.org/10.3923/ajcs.2017.50.62.
Moura, W.S., Ascheri, D.P.R., 2018. Elaboration of Biodegradable Films of *Hedychium coronarium*, 1st ed., Vol. 1. New academic editions, Mauritius, p. 125.
Moura, W.S., Souza, S.R., Campos, F.S., Cangussu, A.S.R., Santos, E.M.S., Andrade, B.S., Viana, K.F., Haddi, K., Oliveira, E.E., Nascimento, V.L., Aguiar, R.W.S., 2020. Antibacterial activity of *Siparuna guianensis* essential oil mediated by impairment of membrane permeability and replication of pathogenic bacteria. Ind. Crops Prod. 46, 1–9. https://doi.org/10.1016/j.indcrop.2020.112142.
Murúa-Pagola, B., Beristain-Guevara, C.I., Martínez-Bustos, F., 2009. Preparation of starch derivatives using reactive extrusion and evaluation of modified starches as shell materials for encapsulation of flavoring agents by spray drying. J. Food Eng. 91 (3), 380–386. https://doi.org/10.1016/j.jfoodeng.2008.09.035.
NIST, Chemistry WebBook, 2018. In: Linstrom, P.J., Mallard, W.G. (Eds.), NIST Standard Reference Database Number 69. National Institute of Standards and Technology, Gaithersburg MD, p. 20899. https://doi.org/10.18434/T4D303.
OECD, 2013. *Test No. 236:* Fish Embryo Acute Toxicity *(FET) Test*, OECD Guidelines for the Testing of Chemicals, Section 2. Organization for Economic Co-operation and Development. OECD Publishing, Paris. https://doi.org/10.1787/9789264203709-en.
Oliveira, M.S., Cruz, J.N., Almeida da Costa, W., Silva, S.G., Brito, M.P., de Menezes, S.A. F., de Jesus Chaves Neto, A.M., de Aguiar Andrade, E.H., de Carvalho Junior, R.N., 2020. Chemical composition, antimicrobial properties of *Siparuna guianensis* essential oil and a molecular docking and dynamics molecular study of its major chemical constituent. Molecules 25, 3852. https://doi.org/10.3390/molecules25173852.
Pavela, R., 2015. Essential oils for the development of eco-friendly mosquito larvicides: a review. Ind. Crops Prod. 76, 174–187. https://doi.org/10.1016/j.indcrop.2015.06.050.
Pavela, R., Benelli, G., Canale, A., Maggi, F., Mártonfi, P., 2020. Exploring essential oils of Slovak medicinal plants for insecticidal activity: the case of Thymus alternans and Teucrium montanum subsp. Jailae. Food Chem. Toxicol. 138, 111203 https://doi.org/10.1016/j.fct.2020.111203.
Peres, M.C., de Souza Costa, G.C., dos Reis, L.E.L., da Silva, L.D., Peixoto, M.F., Alves, C. C.F., de Melo Cazal, C., 2020. In natura and nanoencapsulated essential oils from *Xylopia aromatica* reduce oviposition of *Bemisia tabaci* in *Phaseolus vulgaris*. J. Pest Sci. 93, 807–821. https://doi.org/10.1007/s10340-019-01186-6.
Peterson, D.L., Böröczky, K., Tumlinson, J., Cipollini, D., 2020. Ecological fitting: chemical profiles of plant hosts provide insights on selection cues and preferences for a major buprestid pest. Phytochemistry 176, 112397. https://doi.org/10.1016/j.phytochem.2020.112397.
Portella, A.C.F., Murano, M., Ascêncio, S.D., Siqueira, C.A., Ferreira, T.P.S., Aguiar, R.W. S., 2014. Physical and chemical Characterization of essential oil of *Siparuna guianensis* Aublet. Química Nova Na Esc. 37, 844–849. https://doi.org/10.5935/0100-4042.20140136.
Ribeiro, J.S., Veloso, C.M., 2021. Microencapsulation of natural dyes with biopolymers for application in food: a review. Food Hydrocoll. 112, 106374 https://doi.org/10.1016/j.foodhyd.2020.106374.
Rizzo, R., Lo Verde, G., Sinacori, M., Maggi, F., Cappellacci, L., Petrelli, R., Vittori, S., Morshedlood, M.R., Yvette Fofie, N.G.B., Benelli, G., 2020. Developing green insecticides to manage olive fruit flies? Ingestion toxicity of four essential oils in protein baits on *Bactrocera oleae*. Ind. Crops Prod. 143, 111884 https://doi.org/10.1016/j.indcrop.2019.111884.
Samakradhamrongthai, R., Thakeow, P., Kopermsub, P., Utama-Ang, N., 2015. Encapsulation of *Michelia alba* D.C. Extract using spray drying and freeze drying and application on thai dessert from Rice Flour. IJFER 1 (2). https://doi.org/10.18178/ijfe.1.2.77-85.
Sanjeewa, K.K.A., Kang, N., Ahn, G., Jee, Y., Kim, Y., Jeon, Y., 2018. Bioactive potentials of sulfated polysaccharides isolated from brown seaweed *Sargassum* spp in related to human health applications: a review. Food Hydrocoll. 81, 200–208. https://doi.org/10.1016/j.foodhyd.2018.02.040.
Sun, J.S., Feng, Y., Wang, Y., Li, J., Zou, K., 2020. α-Pinene, Caryophyllene and β-Myrcene from *Peucedanum terebinthaceum* essential oil: insecticidal and repellent effects on three stored-product. Insects Rec. Nat. Prod 14 (3), 177–189. https://doi.org/10.25135/rnp.149.19.05.1287.
Tappiban, P., Smith, D.R., Triwitayakorn, K., Bao, J., 2019. Recent understanding of starch biosynthesis in cassava for quality improvement: a review. Trends Food Sci. Technol. 83, 67–180. https://doi.org/10.1016/j.tifs.2018.11.019.
Tappiban, P., Sraphet, S., Srisawad, N., Wu, P., Han, H., Smith, D.R., Bao, J., Triwitayakorn, K., 2020. Effects of cassava variety and growth location on starch fine structure and physicochemical properties. Food Hydrocoll. 108, 106074 https://doi.org/10.1016/j.foodhyd.2020.106074.
Toledo, P.F.S., Ferreira, T.P., Bastos, I.M.A.S., Rezende, S.M., Jumbo, L.O.V., Didonet, J., Andrade, B.S., Melo, T.S., Smagghe, G., Oliveira, E.E., Aguiar, R.W.S., 2019. Essential oil from Negramina (*Siparuna guianensis*) plants controls aphids without impairing survival and predatory abilities of non-target ladybeetles. Environ. Pollut. 255 (1), 113153. https://doi.org/10.1016/j.envpol.2019.113153.
Tomazelli, O., Kuhn, F., Padilha, P.J.M., Vicente, L.R.M., Costa, S.W., Boligon, A.A., Scapinello, J., Nesi, C.N., Dal Magro, J., Castellví, S.L., 2018. Microencapsulation of essential thyme oil by spray drying and its antimicrobial evaluation against *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. Braz. J. Biol. 78, 2. https://doi.org/10.1590/1519-6984.08716.
Tongdeesoontorn, W., Mauer, L.J., Wongruong, S., Sriburi, P., Rachtanapun, P., 2011. Effect of carboxymethyl celulose concentration on physical properties of biodegradable cassava starch-based films. Chem. Central J. 5 (2011), 6. https://doi.org/10.1186/1752-153X-5-6.
Vicentini, N.M., 2003. Elaboration and Characterization of Edible Films Based on Cassava Starch for Use in Post-harvest. Thesis. UNESP, São Paulo, SP, p. 198.
Zaitoon, A., Lim, L., Scott-Dupree, C., 2021. Activated release of ethyl formate vapor from its precursor encapsulated in ethyl Cellulose/Poly(Ethylene oxide) electrospun nonwovens intended for active packaging of fresh produce. Food Hydrocoll. 112, 106313 https://doi.org/10.1016/j.foodhyd.2020.106313.

DECLARAÇÃO

Declaramos que no dia 08 de Novembro de 2021, o professor Dr. Raimundo Wagner de Souza Aguiar, docente da UFT, CPF: 771.000.851/87, deu entrada junto ao Núcleo de Inovação Tecnológica da Universidade Federal do Tocantins em processo referente a pedido de patente intitulado USO DO EXTRATO DE *Chiococca alba* (L.) Hitchc. e *Siparuna guianensis* Aubl. CONTRA O VÍRUS DA COVID-19. Os inventores listados são: Dr. Wellington de Souza Moura (Aluno de pós graduação da Rede Bionorte - UFT) e Dr. Raimundo Wagner de Souza Aguiar (docente UFT), Dr. Eugênio Eduardo Oliveira (UFV); Bergmann Morais Oliveira (UNB); Dr. Lúcio Holanda Gondim de Freitas Júnior (USP) e Dra. Carolina Borsoi Moraes Holanda de Freitas (UNIFESP).

Declaramos ainda, que o referido pedido se encontra em fase de instrução processual e que somente será depositado junto ao Instituto Nacional de Propriedade Industrial – INPI em caso de cumprimento de todas as instâncias inerentes ao processo, entre elas: entrega de redação, entrega de documentos pessoais dos inventores, comprovação de vínculo dos pesquisadores com as instituições co-titulares, entrega da documentação das instituições co-titulares, assinatura de procurações e contratos de cessão de titularidade e parecer favorável do Comitê de Avaliação de Propriedade Intelectual da UFT quanto à suficiência descritiva, atividade inventiva, aplicação industrial e novidade da tecnologia.

Palmas, 16 de Novembro de 2021.

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