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PSIDIUM CATTLEIANUM SABINE LEAVES EXTRACTS: PHENOLIC COMPOUNDS AND ANTIOXIDANT, ANTIMICROBIAL AND ALLELOPATHIC ACTIVITIES

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Área de Concentração Desenvolvimento e otimização de tecnologias, produtos e processos, da Universidade do Estado de Santa Catarina (UDESC), como requisito parcial para obtenção de grau de Mestre em Ciência e Tecnologia de Alimentos. Orientadora: Dra. Aniela Pinto Kempka. Coorientadora: Dra. Liziane Schittler Moroni.

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MARINA VOLPATO DACOREGGIO

EXTRATOS DAS FOLHAS DE *PSIDIUM CATTLEIANUM* SABINE: COMPOSTOS FENÓLICOS E ATIVIDADES ANTIOXIDANTE, ANTIMICROBIANA E ALELOPÁTICA

Dissertação apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, da Universidade do Estado de Santa Catarina, como requisito parcial para obtenção do grau de Mestre em Ciência e Tecnologia de Alimentos.

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“... e aprendi que se depende sempre, de

tanta, muita, diferente gente...

toda pessoa sempre é as marcas das

lições de outras tantas pessoas...

.. e é tão bonito quando a gente entende

Que a gente é tanta gente

Onde quer que a gente vá...”

(Gonzaguinha)

RESUMO

Psidium cattleianum Sabine (Myrtaceae) é uma planta nativa do sul do Brasil que está sendo considerada uma planta com perspectivas latentes para a indústria farmacêutica e alimentícia. O objetivo do presente estudo foi obter extratos de folhas de *Psidium cattleianum* Sabine, coletadas no verão e no inverno, utilizando ultrassom e enzimas, com o intuito de minimizar a utilização de solventes potencialmente agressivos ao meio ambiente e, caracterizar e avaliar as atividades antioxidante, alelopática e antimicrobiana dos extratos. Para verificar a influência do método de extração na estrutura das folhas, fotomicrografias foram obtidas por microscopia eletrônica de varredura (MEV). Os extratos foram caracterizados quanto ao teor de compostos fenólicos totais e a atividade antioxidante foi verificada pelo efeito da eliminação do radical DPPH. A atividade alelopática foi verificada em bioensaios para cada um dos extratos contra uma planta teste de *Lactuca sativa* cv. Grands rapids. Por fim, a atividade antimicrobiana foi determinada frente a bactérias Gram-positivas (*Staphylococcus aureus* e *Listeria monocytogenes*) e Gram-negativas (*Escherichia coli* e *Salmonella* Enteritidis) de importância patogênica na indústria de alimentos, através da técnica de difusão em disco e Concentração Inibitória Mínima para cada uma das espécies bacterianas. Avaliou-se, ainda, a utilização dos extratos como desinfetantes de diferentes superfícies. Acerca dos extratos obtidos, a MEV mostrou que a forma de extração afetou a estrutura celular das folhas de forma distinta, porém não influenciou no teor total de compostos fenólicos. O maior teor fenólico foi encontrado nos extratos de folhas coletadas durante o inverno (123,02 ± 0,10 mg de EAG/g de material vegetal seco para a extração assistida por ultrassom e 143,80 ± 0,46 mg de EAG/g de material vegetal seco para a extração com auxílio de enzimas). Já o nível de atividade antioxidante estimado apresentou maior valor para as folhas coletadas no verão. Os resultados também indicam que extratos de todas as amostras e extrações testadas apresentam considerável atividade alelopática, com inibição do crescimento superior a 50%. Para as bactérias testadas (Gram-positivas e Gram-negativas), a atividade antimicrobiana pode ser classificada como parcialmente ativa a muito ativa, onde os melhores resultados foram encontrados contra a bactéria *L. monocytogenes*, com halos de inibição medindo até 23 mm. Verificou-se também que a época de coleta das folhas de *P. Cattleianum* Sabine interferiu nos valores encontrados. Em relação a desinfeção de superfícies, todos os extratos testados apresentaram resultados positivos. Os experimentos realizados demonstram que os extratos de *Psidium cattleianum* Sabine são uma fonte de compostos bioativos, dentre os quais se encontram os compostos fenólicos, que possuem atividade antioxidante, alelopática e antibacteriana.

Palavras-chave: *Psidium cattleianum* Sabine. Atividade antioxidante. Atividade alelopática. Atividade antimicrobiana.

ABSTRACT

Psidium cattleianum Sabine (Myrtaceae) is a plant native to southern Brazil that is being considered a plant with latent perspectives for the pharmaceutical and food industry. The objective of the present study was to obtain extracts of leaves of *Psidium cattleianum* Sabine, collected in the summer and winter, using ultrasound and enzymes, in order to minimize the use of potentially aggressive solvents to the environment and, to characterize and evaluate the activities antioxidant, allelopathic and antimicrobial properties of the extracts. To verify the influence of the extraction method on the leaf structure, photomicrographs were obtained by scanning electron microscopy (SEM). The extracts were characterized in terms of the total phenolic compounds content and the antioxidant activity was verified by the effect of the elimination of the DPPH radical. The allelopathic activity was verified in bioassays for each of the extracts against a test plant of *Lactuca sativa* cv. Grands rapids. Finally, the antimicrobial activity was determined against Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) and Gram-negative (*Escherichia coli* and *Salmonella* Enteritidis) bacteria of pathogenic importance in the food industry, through the disc diffusion technique and Inhibitory Concentration Minimum for each of the bacterial species. It was also evaluated the use of extracts as desinfectants of different surfaces. Regarding the extracts obtained, the SEM showed that the extraction form affected the cell structure of the leaves in a different way, but did not influence the total content fo phenolic compounds. The highest phenolic content was found in leaf extracts collected during winter (123.02 ± 0.10 mg EAG / g of dry plant material for ultrasonic assisted extraction and 143,80 ± 0,46 mg EAG / g of dry plant material for the extraction with the aid of enzymes). Already, the level of antioxidant activity estimated presented higher value for the leaves collected in the summer. The results also indicate that extracts from all the samples and extractions tested show considerable allelopathic activity, with inhibition of growth of more than 50%. For the tested bacteria (Gram-positive and Gram-negative), the antimicrobial activity can be classified as partially active to very active, where the best results were found against *L. monocytogenes* bacteria, with inhibition halos measuring up to 23 mm. It was also verified that the time of collection of leaves of *P. cattleianum* Sabine interfered in the values found. With regard to surface disinfection, all the extracts tested positive results results. Experiments have shown that extracts of *Psidium cattleianum* Sabine are a source of bioactive compounds, among which are the possibility of occurrence of phenolic compounds, which have antioxidant, allelopathic and antibacterial activity.

Keywords: Psidium cattleianum Sabine. Antioxidant activity. Allelopathic activity. Antibacterial activity.

SUMMARY

1 GENERAL INTRODUCTION

Brazil has more than 55,000 species of superior plants distributed in five main biomes: Atlantic Forest, Cerrado, Amazon, Pantanal and Pampa, considered the richest in the planet (FIASCHI; PIRANI, 2009; SOUZA *et al*., 2008). Despite the great potential, the number of domesticated native species that is being used as a natural antioxidant or antimicrobial is still limited (MEDINA *et al*., 2011a; PROTEGGENTE *et al*., 2002).

Several plants have been studied for their antioxidant capacity, considering that the interest in replacing the synthetic antioxidants (most commonly used), due to its possible carcinogenic effects (BOTTERWECK *et al*., 2000), by natural antioxidants.

Medicinal plants have a variety of molecules capable of eliminating free radicals, such as phenolic acids, flavonoids, catechins, proanthocyanidins, quinones, coumarins, tannins, terpenoids, carotenoids and other metabolites that have antioxidant activity (BABBAR *et al*., 2014; LIN *et al*., 2016). In addition, they may offer treatment or prevention of infectious diseases without the side effects of synthetic antibiotics (AFTABUDDIN *et al*., 2017).

Another benefit from plants is their allelopathic activity. This activity is able to inhibit or even modify the patterns of growth and development of other plants, serving as natural herbicides in food production (JABRAN *et al*., 2015).

Of the subtropical biomes, the araçá (*Psidium cattleianum* Sabine) appears as an example of a native fruit of great potential (DREHMER; AMARANTE, 2008). Exploratory studies suggest high antioxidant activity and high phenolic content. Furthermore, they demonstrate nutritional, functional and antimicrobial potential (SOUZA *et al*., 2004; GALHO *et al*., 2007; MEDINA *et al*., 2011).

The objective of this work was to obtain the bioactive compounds from leaves of *Psidium cattleianum* Sabine collected in different seasons of the year (winter and summer), using different methods of extraction (ultrasound and enzymes) that aimed at the use of clean and solvent methodologies just the water. As well as, determine the antioxidant, antimicrobial and allelopathic activities for each of the extracts.

His dissertation is divided into three chapters, where Chapter 1 describes the bibliographic review, followed by Chapter 2, which contains the article "Extract of *Psidium cattleianum* Sabine leaves: phenolic compounds, antioxidant and allelopathic activities" containing materials, methods, results, discussion and partial conclusion, and Chapter 3 with

the article "Antimicrobial activity of extract of *Psidium cattleianum* Sabine leaves", containing materials, methods, results, discussion and partial conclusion, General conclusion of the study and Bibliographical References.

2 CHAPTER 1

In this chapter, we discuss relevant issues on the subject with information on *Psidium cattleianum* Sabine, popularly known as araca and its leaves, phenolic compounds and antioxidant, allelopathic and antimicrobial activities.

2.1.1. BIBLIOGRAPHIC REVIEW

The following are topics related to bibliographic review.

2.1.1 *Psidium cattleianum* Sabine

Psidium cattleianum Sabine (Myrtaceae) is a Brazilian native species that can be found from Bahia to the southern states, and also in the neighboring country, Uruguay, besides Hawaii and many Caribbean islands, where it has adapted (PATEL, 2012). Arvoreta or shrub up to 6 meters, presents tortuous trunk, thin bark and reddish chestnut, in addition to simple and opposite leaves (Figure 1). Its flowers are formed in the branches, they have white coloration, while its fruit is a globose, piriforme, ovoid or flattened berry, of yellow or red color when ripe, crowned by the chalice; the pulp may be white, light yellow or red. The size of the fruit varies from 2.2 to 5.0 cm in diameter (CORADIN *et al*., 2011).

Figure 1 - Appearance of the tree, leaves and flower of *Psidium cattleianum* Sabine popularly known as araçá.

Source: Prepared by the author, 2019.

The fruits are known in Brazil, mainly as araçá, araçá-amarelo, araçá-vermelho and araçá-de-praia. Being a succulent fruit, the araçá consumed in natura or processed (sweets, jellies and juices), have high potential for the agri-food sector. In addition, due to the bioactivity (antiproliferative, antidiabetic and antimicrobial) of fruit extract, which may be related to the high content of vitamin C and antioxidants, the araçá may also be valuable for the pharmaceutical industry (BIEGELMEYER *et al*., 2011; MEDINA *et al*., 2011).

Franco (1999) pointed out the presence of retinol, thiamine, riboflavin, niacin, ascorbic acid, sugars, proteins, lipids, calcium, phosphorus, iron and 37.8 kcal in 100 g of fruit. Andrade *et al*. (1993) etermined 85-86% moisture, pH of 3, 1.87% citric acid and 11 ° Brix, in addition to 5.05% sugars, 0.103 mg carotenoids and 389.34 mg vitamin C in 100 g of sample.

Accorgind to Medina *et al*. (2011), due to the ability of the species to adapt to stress conditions, araçá can be considered as a fruit rich in secondary metabolites, therefore possessing interesting functional properties, considering that species that have phenolic compounds, ascorbic acid and carotenoids in considerable amounts are usually associated with important biological properties, such as enhanced protection against cellular oxidation, antimicrobial activity and anticarcinogenic activity.

Thus, when included in the human diet, they contribute to reduce the development of degenerative diseases, such as cancer, cardiovascular diseases, diabetes, among others. In addition, araçá also contains other interesting chemical compounds, such as minerals, fatty acids, sugars, volatile compounds and carotenoids, which can also contribute to human health (PEREIRA *et al*., 2018; VERMA *et al*., 2013).

Its leaves have traditionally been used in some Eastern countries for the treatment of diarrhea and diabetes (PATEL, 2015). Recently, leaf extract has been administered in cancer therapy, pathogenic infections and inflammation in experimental models (IM *et al*., 2012).

Alvarenga *et al*. (2016), when evaluating the chromatographic profile of leaves of *Psidium cattleianum* Sabine, observed the prevalence of compounds such as quercetin, quercitrin and isoquercitrin, all of the class of flavonoids, and ellagic acid, as well as lower amounts of catechin, chlorogenic acid and Kaempferol.

Im *et al*. (2012) investigated the molecular mechanism behind the antimetastatic effects of the butanolic fraction of *P. cattleianum* leaf extract. Collectively, the results indicated that leaf extracts may contain various forms of malignant. Already Brighenti *et al*. (2008)

evaluated the effects of aqueous extract of leaves of *P. cattleianum* on pathogenic *Streptococcus mutans*. They concluded that leaf extract kills most S. mutans when applied at high concentrations, i.e., 25, 50 or 100%. Leaf extract also showed anticary effects in rats (JUN *et al*. 2011).

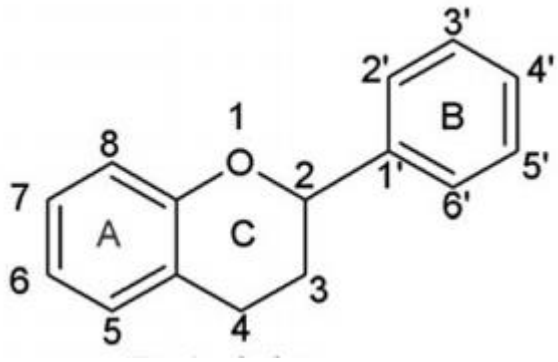
2.1.1. Phenolic Compounds

Phenolic compounds are chemical structures that have hydroxyls and aromatic rings, in simple forms or polymers, which gives them antioxidant power. Originating from the secondary metabolism of plants, they are critical for their growth and reproduction. Its formation occurs in conditions of stress as, injuries, infections, UV radiation, among others. These phenolic substances, or polyphenols, contain numerous varieties of compounds: simple flavonoids, phenolic acids, complex flavonoids and colored anthocyanins (BABBAR *et al*., 2014; LIN *et al*., 2016). hese phenolic compounds are generally related to defense responses in the plant. However, they play an important role in other processes, such as the incorporation of substances that accelerated pollination, coloring for camouflage and defense against herbivores (EDREVA *et al*., 2008).

Present in substantial amounts in plant foods such as legumes, pulses, fruits, nuts and berries and related processed foods (juices, teas and wines), their consumption has been associated with reducing the risk of multiple non-communicable chronic diseases, cardiovascular diseases and neurodegenerative diseases, type II diabetes and osteoporosis, supposedly attributed to its multifaceted bioactivity: anti-oxidation, anti-inflammation, signal transduction modulation, antimicrobial activity and anti-proliferation (VELDERRAIN-RODRÍGUEZ *et al*., 2014).

Flavonoids are considered one of the most significant groups of phenolic antioxidants. Its chemical structure consists of two aromatic rings, called rings A and B, joined by three carbons that form a heterocyclic ring, called ring C (Figure 2). Changes in standard C-ring results in important classes of flavonoids, such as flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, and anthocyanidins. Substitutions of rings A and B give rise to different compounds within each class of flavonoids (ANGELO; JORGE, 2007; SIMONETTI *et al*., 2016).

Figure 2 - Basic structure of flavonoids.



Source: Prepared by the author, 2019.

In vitro studies of the fruits of *Psidium cattleianum* Sabine demonstrated total contents of phenolic compounds and flavonoids, expressed in fresh weight, as more abundant in red araca (501.33 mg of gallic acid equivalents per 100 g) than in yellow (292, 03 mg of gallic acid per 100 g) (BIEGELMEYER *et al*., 2011). However, in other studies, the total content of phenolic compounds did not differ between the yellow and red genotypes, for example 5,372 mg and 5,638 mg of gallic acid (GAE) per 100 g, were reported for yellow araçá and red araçá. (LUXIMON-RAMMA *et al*., 2003; VINHOLES *et al*., 2017).

2.1.2. Atioxidant activity

In the food industry, an antioxidant is defined as a substance that, in small amounts, is capable of significantly preventing or retarding the oxidation of readily oxidizable materials (MIGUEL, 2010). Oxidation is a chemical reaction that transfers electrons or hydrogen from substances to an oxidizing agent. Oxidation reactions can produce free radicals, which in turn can initiate chain reactions. When chain reactions occur in a cell, they can cause damage or even death to the cell (PISOSCHI; NEGULESCU, 2012).

Antioxidants are responsible for the body's defense mechanisms against the pathologies associated with free radical attack. Thus, consumption of plant-derived antioxidants is involved in the prevention of degenerative diseases caused by oxidative stress such as cancer, Parkinson's, Alzheimer's or atherosclerosis (LEE; KOO; MIN, 2004; VALKO *et al*., 2007).

Natural defense systems include a variety of substances that act on three different levels of the oxidative process: (1) blocking the initiation stage because they prevent the generation of reactive species or sequester them in a way that prevents their interaction with cellular targets , such as tocopherols, carotenoids and antioxidant enzymes; (2) blocking the radical chain progression stage by sequestering intermediate radicals, such as flavonoids and synthetic antioxidants; and finally (3) repairing the lesions caused by reactive oxygen metabolism species (ROS) (DELTON-VANDENBROUCKE *et al*., 2001; NIKI, 2010).

Antioxidants can be classified into: primary antioxidants, those that interrupt the chain of reactions involved in lipid oxidation by donating electrons or hydrogen to free radicals, converting them into more stable, and secondary antioxidants, those compounds that reduce or retard the rate of initiation of oxidation by decomposition of hydroperoxides (SHAHIDI; NACZK, 2003).

Several are the synthetic antioxidant agents available. However, the toxic and collateral effects in animals and humans is increasing; as is the case of butylhydroxytoluene (BHT) and 2-tert-butyl-4-hydroxyanisole / 3-tert-butyl-4-hydroxyanisole (BHA) which have carcinogenic potential (BOTTERWECK *et al*., 2000; RIBEIRO *et al*., 2014)

Substitution of synthetic antioxidants with natural antioxidants may have advantages because of health and functional implications. It is noted, for example, that the increased solubility of the natural antioxidants in both water and oil is useful in the preparation of emulsions and other formulations, such as hydrogels (OLIVEIRA *et al*., 2009).

In vitro studies indicate that *P. cattleianum* can be considered as a good source of bioactive compounds with antioxidant properties (MCCOOK-RUSSELL *et al*., 2012; DALLA NORA *et al*., 2014). According Ribeiro *et al*. (2014), araçá is a potent hijacker of reactive species of oxygen and nitrogen. Vinholes *et al*. (2017) reported similar results for edible portions of yellow and red genotypes that showed good inhibition properties. Results showed that the yellow araca (IC50 = 334.3 $\mu g.mL^{-1} \pm 16.5 \mu g.mL^{-1}$) was more effective against DPPH than the red genotype (IC50 = 490.3 $\mu g.mL^{-1} \pm 35.1 \mu g.mL^{-1}$).

2.1.3. Allelopathic activity

Allelopathy can be defined as the positive or negative interference that chemical compounds produced by a plant exert on other organisms (plants, fungi, algae and insects);

where almost all parts of the plant can contain such compounds (allelochemicals), with allelopathic activity (FERREIRA; BORGHETTI, 2004).

The allelochemicals possibly originate from the primary or secondary metabolism of plants since both have their production regulated by environmental factors, among them, temperature, light intensity, availability of water and nutrients, soil texture and microorganisms present (CARMO; BORGES; TAKAKI, 2007). In addition, stress-related factors may increase the biological activity of these substances (INDERJIT; CALLAWAY; VIVANCO, 2006).

Almeida *et al*. (2008) have indicated some mechanisms of action of the allelochemicals, which can affect the processes of photosynthesis, respiration, enzymatic activity, water relations, stomatal opening, cell division and stretching. However, they realized that many of these processes occur due to oxidative stress, that is, an oxygen molecule in its diatomic state, when accepting an electron, forms a superoxide. This process runs in plant tissues, where by the action of some enzymes, the superoxide radical is transformed into water. The allelochemical can then inhibit the enzyme fundamental to the production of water, causing the death of the plant.

Many allelochemicals have been identified, but most allelopathy studies use plant extracts containing unknown compounds. In the majority, the allelochemicals are secondary metabolites of the plant belonging to: terpenoids, phenolic compounds, long chain fatty acids, organic cyanides and others (GNIAZDOWSKA; BOGATEK, 2005). The action of these compounds is mainly involved in the inhibition and modification of plant growth or development.

The Board 1shows some chemical classes that deserve more attention in relation to their allelopathic potential.

Board 1 – Main classes of secondary metabolites with allelopathic activity and their mechanisms of action.

PHYTOCHEMICAL	**MECHANISM**	**REFERENCES**
Alkaloids	They inhibit the growth of bacteria, as well as being toxic to some invertebrates	(INDERJIT; CALLAWA; VIVANCO, 2006)

Flavonoids	Inhibit the growth of plants and fungi	(SHIMOJI; YAMASAKI, 2005)
Tannins	Proteins bind, forming precipitates that have astringent taste and difficult digestion, protecting against the attacks of vertebrate herbivores and invertebrates	(FERREIRA; AQUILA, 2000)
Terpenoids	They act as growth regulators, phytoalexins and repellents for herbivorous insects	(MAIRESSE *et al.*, 2007)
Phenolics	They act in the defense against herbivores and pathogens, in the attraction of pollinators, in the protection with UV light and in the establishment of the symbiosis	(ALVES *et al*., 2004; CARMO; BORGES; TAKAKI, 2007)

In Hawaii, since it was introduced in 1825 as an ornamental garden plant, *P. cattleianum* became an invasive species, which unleashed serious problems (PATEL, 2012). Its uncontrollable growth was explained by the production of toxic chemicals by its leaves, such as chlorogenic acid, quercetin, catechin and ellagic acid (REZENDE *et al*., 2003), which prevents the growth of other species (GERLACH, 2004), showing an allelopathic potential, which is characterized by the positive or negative interference that the chemical compounds produced by a plant exert on other organisms (RICE *et al*., 1984).

Hister *et al*. (2016) reported that the aqueous extract of leaves of *Psidium cattleianum* showed a decrease in seed germination of Lactuca sativa L. in relation to the negative control,

as well as the extracts that presented concentration of 75 $g.L^{-1}$ of dry leaves showed partial inhibition to total germination.

2.1.4. Antimicrobial activity

Infectious diseases are the world's leading cause of premature deaths, where morbidity and mortality from diarrhea remain a major problem in many developing countries. Infections due to the variety of bacterial etiological agents, such as *Escherichia coli*, *Salmonella spp*., *Staphylococcus aureus* are very common (ALAVIJEH; SHARMA, 2012).

Antimicrobials or antibiotics are drugs used to cause death or inhibit the growth of a particular microbial agent. They originate from substances produced by the microorganisms themselves, originating from plants or even synthetic (ALTERTHUM; TRABULSI, 2003).

Resistance to human drugs by pathogenic bacteria has been commonly reported worldwide. A bacterium is considered resistant to a given antibiotic when it is able to grow in vitro in the presence of the minimal inhibitory concentration of this drug. Resistance may be natural, where bacteria genes encode enzymes that inactivate the mechanism of action of the drug; or acquired, when the bacterium becomes resistant to a sensitive drug, through the acquisition of genetic factors or mutation in its defense genes (TAVARES, 2006).

Several technological measures have been suggested in order to solve the problem of resistance of bacteria, one of them being the search for new antimicrobials from plant species. There are several reports on the antimicrobial activity of different plant extracts (BOER *et al*., 2005; KEYHANFAR *et al*., 2004).

Many plants studied have the capacity to treat urinary infections, gastrointestinal disorders, respiratory diseases and cutaneous infections (BRANTNER; GREIN, 1994; SOMCHIT *et al*., 2003). Compounds isolated from plants are substances whose chemical structure, with rare exceptions, exhibits large structural differences in relation to antibiotics derived from microorganisms (ALAVIJEH; SHARMA, 2012).

The main groups of compounds with antimicrobial properties extracted from plants include terpenoids, tannin, coumarin, essential oils, alkaloids, lectins, polypeptides and phenolic substances and polyphenols, which are: phenolic acids, simple phenols, quinones, flavonols, flavones and flavonoids (COWAN, 1999; NCUBE; AFOLAYAN; OKOH, 2008).

Some mechanisms responsible for the antimicrobial action of the active principles of plants have already been studied and are related to the regulation of the intermediate metabolism of the bacteria through the blockade of chemical reactions, the direct action on the enzymatic synthesis, or even alterations in the membrane structures (TIWARI *et al*., 2011).

The Board 2 summarizes the main mechanisms of action of plant antimicrobials according to the classes and subclasses of secondary metabolites already studied.

Board 2 – Main classes and subclasses of secondary metabolites with antimicrobial activity and their mechanisms of action.

CLASS	***SUBCLASS***	**MECHANISM OF ACTION**
Alkaloids	ND*	Ability to intercalate with DNA.
Phenolics	Simple phenols and phenolic acids	Enzymatic inhibition by oxidized compounds, possibly through the reaction with sulfhydryl groups.
	Quinones	Bonding with adhesins; Inactivation of proteins and consequent loss of function; Deprivation of substrates for the microorganism.
	Flavones, flavonoids and flavonols	Complexation with extracellular and soluble proteins, as well as bacterial cell wall.
	Tannins	Substrate deprivation; Inactivation of microbial adhesins and enzymes.
Terpenoids	ND	Disintegration of the membrane by lipophilic compounds.

* ND: do not have subclass.

Source: Adapted from Prashant Tiwari *et al*. (2011).

Fruit extracts from *Psidium cattleianum* Sabine demonstrated in vitro antibacterial activity against *Salmonella* Enteritidis, an enteric food-borne pathogen, frequently described in the literature on the occurrence of toxinfections in humans. The extracts had a minimum inhibitory concentration of 5% and extracts with higher concentrations of secondary metabolites were more effective against bacterial proliferation (MEDINA *et al*., 2011).

Intermediate activity has also been reported for araca against *Bacillus subtilis* and *Staphylococcus aureus* (McCOOK-RUSSELL *et al*., 2012).

3. OBJECTIVES

The following are the objectives that guided the research.

3.1. GENERAL

Obtain extracts from the leaves of *Psidium cattleianum* Sabine collected in summer and winter and determine antioxidant, allelopathic and antimicrobial activities.

3.1.1. Specific

- Obtain extracts from the leaves, using as extraction methods the use of ultrasound and the aid of enzymes, separately, in aqueous medium, characterized by being less aggressive methods to the environment;
- Determine the total phenolic compounds content of each extract;
- Determine the antioxidant activity for each of the extracts by the DPPH radical sequestration method;
- Evaluate the allelopathic activity of each extract against the test plant *Lactuca sativa cv*. Grands rapids;
- Determine the antimicrobial activity against Gram-positive and Gram-negative bacteria of pathogenic importance, for each of the extracts;
- Determine the Minimum Inhibitory Concentration for extracts with antimicrobial activity;
- Evaluate the use of the extracts as disinfectants of different surfaces.

4. CHAPTER 2

In this chapter the scientific paper entitled "*Extract of Psidium cattleianum Sabine leaves: phenolic compounds, antioxidant and allelopathic activities*" is presented. The article brings an introduction about the topic, the materials and methods used for the development of the research, the results and discussions, the conclusion. The bibliographic references used are presented at the end of the dissertation.

4.1. INTRODUCTION

The *Psidium* genus comprises 100 spontaneous species, defined as species that produce edible fruits, lumber, and ornamental ones, with the potential for commercial exploration, considered as medicinal plants, which are used in Brazilian traditional medicine to combat oral, gastrointestinal, urogenital and intestinal inflammations (MEDINA *et al*., 2011). Among them, the *Psidium cattleianum* Sabine (Myrtaceae) species, commonly known as Cattley guava, is being considered a plant with latent perspectives for the pharmaceutical and food industry due to its potential application as herbal, functional food, among others (PATEL, 2012), most probably due to the presence of bioactive compounds, such as phenolic compounds and carotenoids (MEDINA *et al*., 2011). It is a Brazilian species that can be found in Bahia, and in the states of Rio Grande do Sul and Santa Catarina, as well as in the neighboring country, Uruguay (BIEGELMEYER *et al*., 2011; PATEL, 2012).

The fruits of *P. cattleianum* have a firm and sweet to sub-acid pulp, with a spicy flavor, and are described as being more aromatic than the common guava fruits, *Psidium* guajava (BIEGELMEYER *et al*., 2011; McCOOK-RUSSEL *et al*., 2012). It has three to four times more ascorbic acid than citric fruits, and it has a great amount of phenolic compounds, such as epicatechin and gallic acid (ALVARENGA *et al*., 2013). Besides its fruits being consumed in natura, experiments with extracts from its leaves have demonstrated antiproliferative activity in cancer cells, as well as antioxidant activity in relation to the radicals DPPH, FRAP, and ABTS, and antimicrobial activity for microorganisms *Streptococcus mutans*, *Salmonella* Enteritidis, *Staphylococcus epidermidis*, *Bacillus subtilis* and *Micrococcus luteus* (BIEGELMEYER *et al*., 2011; BRIGHENTI *et al*., 2008; PEREIRA *et al*., 2018; MEDINA *et al*., 2011; PATEL, 2012; SOUZA *et al*., 2004; VERMA *et al*., 2013).

Cattley guava is a potent captor of species reactive to oxygen and nitrogen, besides its pulp also having inhibiting potential for O_2, HOCl, and $1O_2$ (RIBEIRO *et al*., 2014). Similar

results were obtained by Vinholes *et al.* (2017), in which edible portions of Cattley guava have shown inhibiting properties against O_2^- and hydroxyl radicals.

In Hawaii, since it was introduced, in 1825, as an ornamental garden plant, *P. cattleianum* has become an invading species, which has triggered serious problems (PATEL, 2012). Its uncontrollable growth was explained by the production of toxic chemicals by its leaves, as the chlorogenic acid, quercetin, catechin, and ellagic acid (REZENDE *et al.*, 2003), which prevents the growth of other species (GERLACH, 2004), showing a allelopathic potential, which is characterized by the positive or negative interference that chemical compounds produced by a plant exert over other organisms (RICE *et al.*, 1984).

Plants usually produce numerous secondary metabolites, and some of them have allelopathic activity, specially phenolic ad terpenoid ones (EINHELING, 2002), also produced by *P. cattleianum*. Allelopathic substances may provide a competitive advantage for hosting plants through the inhibition of the growth of competitor plants (KATO-NOGUCHI *et al.*, 2013). Many synthetic agrochemicals are highly toxic to men and other animals, be it due to direct exposition or to the accumulation in the organism after eating contaminated foods. In this sense, allelopathy can be very important, since it enables the identification of compounds, which may serve as basis for the production of more specific herbicides that are also less prejudicial to the environment when compared to the ones being currently used in agriculture (CHRISTOFFOLETI *et al.*, 1994; SILVA *et al.*, 2009).

Given that, this work aims to evaluate the total phenolic contents and its profile, and to determine the antioxidant and allelopathic activity of aqueous extracts of *P. cattleianum* Sabine, obtained with the use of low-frequency ultrasound or enzymes (cellulase complex).

4.2. MATERIALS AND METHODS

The following are the materials and methods used in the research.

4.2.1. Chemicals

The reagents used in this study were high purity ones, having acquired the gallic acid, DPPH (2,2-Diphenyl1-1-Picrylhydrazyl), and Folin-Ciocalteu from Sigma-Aldrich; the sodium carbonate (NA_2CO_3) was acquired from Neon commercial and the methyl alcohol (CH_3OH_3) from Reatec.

4.2.2. Plant material and sample preparation

Cattley guava leaves (*Psidium cattleianum* Sabine), yellow morphotype, were collected in the southern region of the State of Santa Catarina (28°19'31.9" a 28°19'36.5" S; 49°03'50.3" a 49°03'51.9" W), from July to September 2017 (winter) and from December to March 2018 (summer). Leaves were selected according to uniform coloration, ruling out vegetal material with rottenness, injuries and/or defects. The vegetal material was sanitized with gauze dampened with distilled water and dried in a forced circulation stove with air at 40 ±5ºC, to constant mass. After drying, the leaves were manually and separately milled, and sieved in 8 Mesh netting, which finally obtained the samples for extraction.

4.2.3. Preparation and obtaining the extracts

The procedure to obtain the extracts was adapted from Larrauri *et al*. (1997). For the extraction, 15 g of leaves collected in summer and winter were used, separately, and two method were used of each fraction, WU extraction (water + ultrasound) and WE extraction (water + enzyme), for both seasons, winter and summer, in a total of 4 aqueous extracts.

To obtain WU extracts, 100 mL of distilled water was added to the leaf samples, separately. After that, the mix was taken to a ultrasound bath (70 W) for 3 hrs. This mixture stayed at rest, in the dark, for other 3 hrs. The supernatant was filtered with quantitative filter paper ('Whatman' no. 40), and stocked in a volumetric flask of 100 mL, involved in foil paper. To obtain WE extracts, 100 mL of distilled water was added to leaf samples, separately, and 20 µL of a cellulase complex (Novozymes 22086) was added to the mixture. The solution was taken to a bath with orbital agitation at 100 rpm and 45ºC for 6 hrs. The supernatant was filtered with quantitative filter paper ('Whatman' no. 40) and stored in a volumetric flask of 100 mL, involved in foil paper.

Right after the extraction, the extracts were stored in Eppendorf tubes and kept frozen at -83 ºC until its use.

4.2.4. Scanning electron microscopy (SEM)

In order to verify the influence of the extraction method on the structure of leaves, photomicrographs were obtained using scanning electron microscopy (SEM) (microscope

Philips, model XL30). The gold coating was carried out in a BAL-TEC Sputter Coater, model SCD 005, for 120s on the dried leaves (leaf samples were previously dried at 30 ºC for 24 hrs).

4.2.5. Determination of total phenolic content (TPC) and antioxidant activity by elimination of radicals by DPPH

The quantification of TPC was carried out by the Folin-Ciocalteu colorimetric method, with modifications (BONOLI *et al*., 2004). A part of each diluted sample was mixed with 0.5 mL of Folin-Ciocalteu and agitated for 1 min. 2 mL of sodium carbonate (20%) was added to the mixture, and agitated for 30 s. After a 2 hrs incubation in darkness, the absorbance at 750 nm was read in relation to prepared white. The standard curve was prepared by solutions of gallic acid in methanol. The concentration of total phenolic compounds was determined in the extracts as equivalents to gallic acid using an equation obtained based on the standard gallic acid graph and expressed in mg of gallic acid equivalent per dry sample g (mg ECA.g^{-1}). The data were presented as the average of analyses ± standard deviation (SD) of triplicates.

4.2.6. Allelopathic activity

To evaluate the allelopathic effect of the extracts, we performed germination inhibition and growth biotests, according to Formagio *et al*. (2010), with some adaptations, using lettuce (*Lactuca sativa* cv. Grands rapids), as a bioindicator plant. Lettuce seeds were obtained commercially, and non-germinated seeds were used for biogermination tests, while seeds that were germinated 24h before in distilled water were used for growth tests.

For the germination biotests, 10 seeds per replication (plate) were used, as well as 2 replications per treatment, for WU and WE extract for leaves collected in winter and summer, in a total sum of four treatments. The seeds were distributed in Petri dishes (9 cm of diameter) covered with Whatman filter paper no. 2 and damped with 5 mL of solution of the different extracts. 5 mL of distilled water were used in the Control experiment. After putting the seeds on the dishes, they were closed with Parafilm®. Dishes were kept in a BOD oven with 40% humidity and controlled photoperiod, for 5 days. The germination percentage (%G) was obtained by the relation between the number of germinated seeds at the end of the test and the number of seeds added to the dish, the germination index (GI) by the relation between the sum of germinated plantlets in each count and the number of count days, the mean germination time

(MGT) by the relation between the sum of the multiplication of germinated seeds in n each count and the time passed between each count and the number of germinated seeds in each count, and the average speed of germination (ASG) by the relation between 1 and the average germination time.

For the growth biotest, plantlets had their aerial and radicular parts measured with the help of a digital pachymeter and, later on, the plantlets that presented 2 mm of radicle were transferred for new Petri dishes (9 cm of diameter), covered with two Whatman filter paper no. 2 and dampened with 5 mL of the aqueous extracts or distilled water (Control). Petri dishes were incubated in BOD ovens with 40% humidity and controlled photoperiod for 5 days and, having the sizes of plantlets and radicles, the percentage of inhibition of growth (%IG) was calculated in relation to positive control (plants with distilled water).

4.2.7. Statistical analysis

The statistical analysis of experimental results was performed using the software Statistica® 10.0 Statsoft Inc.), performing the Tukey Test, with 95% of reliability.

4.3. RESULTS AND DISCUSSION

Following are the results found in the research and the discussion of the same.

4.3.1. Total phenolic content, antioxidant in relation to DPPH radical and phenolic compounds profile

The results obtained in the determination of the total phenolic content (TPC) expressed as equivalents to gallic acid (EGA) per g of dry vegetal material, and the antioxidant activity (IC_{50}) in relation to DPPH radical, expressed in ($\mu g.mL^{-1}$) of the aqueous extracts of *Psidium cattleianum* Sabine leaves, are shown in Table 1.

Table 1 - Total phenolic content (TPC) and antioxidant activity (IC50) of aqueous extracts of *Psidium cattleianum* Sabine leaves collected in summer and winter.

Leaves	TPC (mg de EGA. g^{-1})		Antioxidant activity DPPH (IC_{50} µg.mL^{-1})	
	WU	**WE**	**WU**	**WE**
Summer	100.62 ± 0.16^{bA}	120.79 ± 0.06^{bA}	37.31 ± 0.89^{bB}	159.63 ± 0.47^{bA}
Winter	143.80 ± 0.46^{aA}	123.02 ± 0.10^{aA}	340.35 ± 1.86^{aA}	208.22 ± 5.32^{aB}

Tukey Test for TPC and antioxidant activity, separated. Different lowercase letters (in the column) correspond to different experiments ($p<0.05$), when comparing the seasons when the leaves were collected. Different uppercase letters (in line), correspond to different experiments ($p<0.05$), when comparing extraction methods. WU and WE correspond to extraction methods using water+ultrasound and water+enzyme, respectively. Source: Prepared by the author, 2019.

For TPC values, it was verified that, both for leaves collected in summer and in winter, there was no statistically significant difference ($p<0.05$) between WU and WE extracts, which shows that the form of extraction did not affect TPC. However, when one verifies the season in which *Psidium cattleianum* Sabine leaves were collected, one notices that it affected the total phenolic content. For the WU extraction, it was verified a statistically significant difference ($p<0.05$) in TPC values for the extracts obtained from the leaves collected in summer and winter, with higher TPC values for the ones collected in winter. The same behavior was observed for WE extractions. Chen *et al*. (2007), when determining the TPC in aqueous extracts of *Psidium gaujaya* L. leaves, have observed values of 154.36 ±2.97 µg of EAG.g^{-1}. Similar values were also observed by Qian and Nihorimbere (2004), in which the TPC value found was 511.6 ±6.2 µg of EAG.g^{-1}.

Gobbo-Neto and Lopes (2007) highlight that there is an influence of harvest season for leaves of a species on the TPC, as well as on its active constituents. It was reported seasonal variation on the contents of almost all classes of secondary metabolites, such as phenolic acids, flavonoids, coumarins, and saponins. Annual, monthly, and even daily temperature variations are one of the factors exerting higher influence of the development of each species, affecting, thus, the production of secondary metabolites. Lower temperatures, a typical characteristic of winter, significantly affects the level of secondary metabolites (HARRIS, 2009). A positive correlation between cold intensity and duration over corn seedlings (*Zea mays*) was reported, as well as the abundance of anthocyanins (CHRISTIE; ALFENITO; WALBOT, 1994). It was also demonstrated, in tobacco leaves (*Nicotiana tabacum*), a four to five times increase in the

content of scopolin, chlorogenic acid, and its isomers (antioxidant compounds) after the plant's exposition to lower temperatures. The increase in chlorogenic acids and anthocyanins, associated to low temperatures, was also observed in *Mahonia repen*s leaves (GOBBO-NETO; LOPES, 2007). Besides temperature, winter has also other characteristics that determine its positive influence in relation to the production and composition of secondary metabolites. The hydrological stress can be mentioned, considering that, in this season, rain is less abundant, which can increase evapotranspiration in species. There are many reports of these conditions leading to an increase in the production of many metabolite types (HARRIS, 2009), as in *Hypericum perforatum*, in which a significant increase in the concentration of flavonoids, hypericins, and chlorogenic acid in flowers under hydrological stress was observed (GRAY *et al*., 2003).

Regarding the antioxidant activity, that is, the extracts ability in capturing free radicals, expressed in the value of IC_{50}, it was verified that, for extracts obtained through the WU method, there was a statistically significant difference ($p<0.05$) in IC_{50} values, when compared leaves collected in winter and summer, with higher antioxidant activity for extracts of leaves collected in summer. The same behavior happens for extracts obtained by the WE method. When comparing extraction methods, for a same season, it was verified that there was statistically significant difference ($p<0.05$) for extracts WU and WE, indicating that the extraction method may affect the availability of molecules with this activity in the extract. For summer leaves, the WU extraction presented higher antioxidant activity, and for winter leaves, the WE extraction presented higher antioxidant activity.

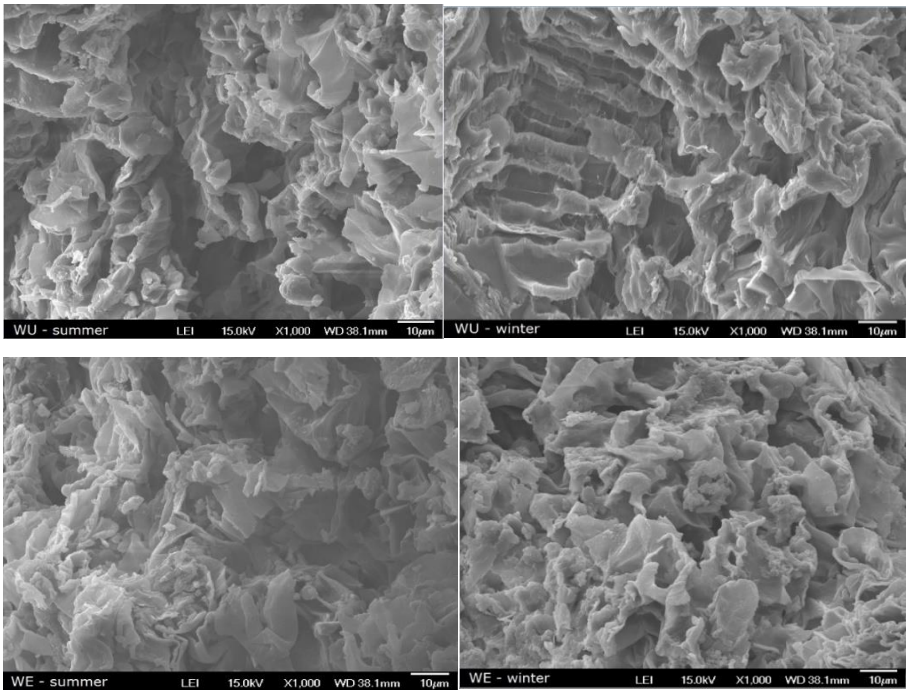
For IC_{50} values lower than 50 $\mu g.mL^{-1}$, the extract is considered very active, for values from 50 - 100 $\mu g.mL^{-1}$, it is considered moderately active, for values between 100 - 200 $\mu g.mL^{-1}$, it is considered slightly active, and for values above 200 $\mu g.mL^{-1}$, it is considered inactive (REYNERTSON *et al*., 2005). Therefore, the extract obtained by the WU method for summer leaves can be considered very active, the extract obtained by the WE method for summer leaves can be considered slightly active, and the extracts obtained by WE and WU methods for winter leaves can be considered inactive.

Qian and Nihorimbere (2004), when studying the aqueous extract of *Psidium guajava* L., obtained the IC_{50} of 130 ±1.0 $\mu g.mL^{-1}$. While Leite et al. (2014), when determining the IC_{50} of hydroalcoholic extracts of *Psidium guajava* L. leaves, obtained the value of 268.40 $\mu g.mL^{-1}$.

The method of determining antioxidant activity was quantified mainly to indicate the antioxidant potential of phenolic compounds isolated in extracts and various substances (BORGES et al., 2011). Waterman and Mole (1994) have found a well-stablished positive correlation between the intensity of solar radiation, higher in summer, and the production of phenolic compounds. In the case of flavonoids and phenylpropanoids, specially, the protection against photo-destruction performed by these metabolites when absorbing and/or dissipating solar energy hampers the damage to more internal tissue by UV-B radiation. Specially for flavonoids, which are accumulated mainly in superficial tissues (such as epidermis, subepidermis, body hair, cuticle, and epicuticular material), and are, thus, used by the plant as UV filters, since they absorb UV-B radiation without changing photosynthetically active radiation. This correlation can be one of the reasons why, even with lower phenolic content values, aqueous extracts of leaves collected in summer have presented higher antioxidant activity than the ones collected during winter.

Figure 3 shows the SEM of the leaves submitted to extraction using ultrasound and the enzyme complex.

Figure 3 - SEM of the leaves submitted to extraction using ultrasound (WU – summer and WU – winter) and the enzyme complex (WE – Summer and WE – winter).



Source: Prepared by the author, 2019.

Differently from that obtained for TPC, it was observed that there was a difference between WU and WE extracts, which shows that the form of extraction did affect the cellular

structure of leaves. For the WU extraction, the formation of cavities in the vegetal structure was verified, which possibly occurred due to the phenomenon of cavitation. US waves induce alternative cycles of compression and rarefaction of the material. Furthermore, when sonication is carried out in a liquid medium, thousands of gaseous cavitation bubbles are formed and part of them collapse which induce alteration of surface tension and viscosity. A fountain of little bubbles moves very fast through material leading to formation of microscopic channels, elongation and flattening of the cells (FERNANDES *et al*., 2008; NOWACKA *et al*., 2012). It is known that the US treatment is connected with free radical generation (KOHNO *et al*., 2011), what can change both chemical composition and antioxidative potential of dried herbs. Santacatalina *et al*. (2014) suggested, the mechanical stress affected by application of US may contribute in releasing of oxidative enzymes and intracellular compounds into the solvent and therefore resulting in phenolic degradation.

For the WE extraction, the occurrence of hydrolysis of the plant structure was observed. Since the cell wall is composed of different polysaccharides bound to a structural protein, mixtures of enzymes and complexes with multiple activity are more efficient than isolated enzymes (JORDAN *et al.,* 2012). The enzymatic complex used is composed of cellulases, which are enzymes classified as glycosyl hydrolases and which degrade the plant cell wall, hydrolyzing oligosaccharides and polysaccharides and recognizing the α-1,4 bonds between glucose molecules (RAVEN *et al*., 2001). Miron *et al*. (2013) compared enzyme-assisted extraction with conventional extraction of phenolic compounds from lemon balm. Cellulase, pectinase and endo-β-1,4-xylanase were used to degrade the cell wall and release the phenolic compounds. The results indicated that enzymatic assisted extraction showed extracts with higher total phenol contents and antioxidant activity compared to the traditional method.

Alvarenga *et al*. (2016) have assessed the chromatographic profile of yellow cattley guava leaves and observed the prevalence of compounds such as quercetin, quercitrin, and isoquercitrin, all part of the flavonoid class, and ellagic acid, as well as lower amounts of catechin, chlorogenic acid and kaempferol. Such compounds can be correlated to the studies aforementioned, suggesting that the higher phenolic content presented in *Psidium cattleianum* Sabine leaves collected in winter may have happened due to relations and factors similar to the ones discussed.

4.3.2. Allelopathic activity in leaf extracts

The evaluation of allelopathic activity if isolated substances or plant extracts can be carried out by the test model of vegetal germination. The observation of changes in seed germination rates shows the toxic and/or cytotoxic action of the tested extracts or substances (LUZ *et al*., 2012). In Table 2, are presented the results of the germination percentage (%G), germination speed index (GI), medium germination time (MGT) and average speed of germination (ASG) of *Lactuca sativa* cv. Grands rapids seeds submitted to aqueous extracts of *Psidium cattleianum* Sabine leaves collected in summer and winter.

Table 2 - Germination percentage (%G), germination speed index (GI), medium germination time (MGT), and average speed of germination (ASG) of Lactuca sativa cv. Grands rapids seed in relation to aqueous extracts of *Psidium cattleianum* Sabine leaves collected in summer and winter

Leaves	**WU**	**WE**
	%G	
Summer	75.00 ± 21.21^{aA}	75.00 ± 7.07^{bA}
Winter	40.00 ± 14.14^{bA}	50.00 ± 0.00^{cA}
Control	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
	GI	
Summer	7.39 ± 3.06^{bA}	3.86 ± 1.20^{aA}
Winter	3.15 ± 1.98^{bB}	16.05 ± 0.71^{bA}
Control	15.33 ± 0.006^{aA}	15.33 ± 0.006^{bA}
	MGT (day)	
Summer	5.15 ± 0.22^{aA}	5.52 ± 1.83^{aA}
Winter	5.39 ± 0.23^{aA}	5.43 ± 0.14^{aA}
Control	4.59 ± 0.01^{aA}	4.59 ± 0.01^{aA}
	ASG (day^{-1})	
Summer	0.194 ± 0.008^{aA}	0.192 ± 0.063^{aA}
Winter	0.186 ± 0.008^{aA}	0.184 ± 0.005^{aA}
Control	0.220 ± 0.003^{aA}	0.220 ± 0.003^{aA}

Tukey Test for %G, GI, MGT and ASG, separated. Different lowercase letters (in the column) correspond to different experiments ($p<0.05$), when comparing the seasons when the leaves were collected and control. Different uppercase letters (in line), correspond to different experiments ($p<0.05$), when comparing extraction methods. WU and WE correspond to extraction methods using water+ultrasound and water+enzyme, respectively. Source: Prepared by the author, 2019.

Regarding the %G for WU extracts, it was verified a statistically significant difference ($p<0.05$) between the extract of winter leaves and the rest, with a 60% inhibition of germination.

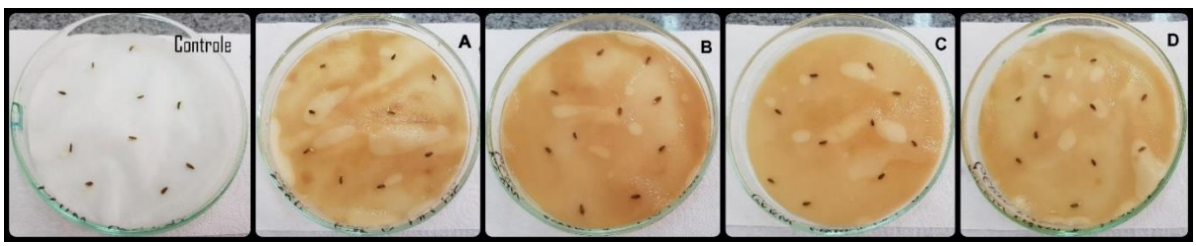
For WE extracts, there was a statistically significant difference ($p<0.05$) among all three extracts, summer leaves, winter leaves, and control. When comparing WU and WE extracts, for the same season, it can be verified that the results did not show significant differences, which demonstrates that the extraction manner did not affect the content of compounds responsible for germination. Thus, the influence of germination happened due to the season when the leaves were collected, with a 25% to 60% variation in germination inhibition, when compared to the control experiment (100%).

For the GI, while summer leaves did not show significant difference ($p>0.05$) in relation to the type of extract used, winter leaves have shown a higher and statistically different germination speed index ($p<0.05$) when the WE extract was used, that is, for this last one, the speed of seed germination can by faster. However, in relation to the season, the WU extract was statistically different ($p<0.05$) when compared both seasons and the control group, while for the WE extract, only summer leaves have shown a statistically different value in relation to the other season and the control. It can be concluded that the reduction of GI can create a delay in the germination process, but not necessarily, after 24 h, a lower %G and consequent increase in the inhibition percentage.

In relation to MGT and ASG, it is observed that the values found were not statistically different ($p>0.05$) regarding the extract used, WU and WE, as well as the seasons and the control experiment. That is, regardless of the extract of season when leaves were collected, Psidium cattleianum Sabine leaves will present stable germination speed and average time.

Figure 4 shows the germination of *Lactuca sativa cv*. Grands rapids seeds in aqueous extracts of *Psidium cattleianum* Sabine leaves collected in summer and winter and in the control experiment, after 24 hours.

Figure 4 – Germination of *Lactuca sativa cv*. Grands rapids seeds in aqueous extracts of *Psidium cattleianum* Sabine leaves collected in summer and winter and the control experiment, after 24 hours.



A = WU extract of summer leaves, B = WE extract of summer leaves, C = WU extract of winter leaves, and D = WE extract of winter leaves. WU and WE correspond to the extraction methods using water+ultrasound and

water+enzyme, respectively. In the control experiment, distilled water was used for wetting. Source: Prepared by the author, 2019.

Hister *et al*. (2016) reported that the aqueous extract of *Psidium cattleianum* leaves have presented a drop in seed germination in relation to the negative control, as well as the extracts that had a concentration of 75 $g.L^{-1}$ of dry leaves presented a partial to total inhibition of germination. Tur *et al*. (2012) also verified that extracts of fresh and dry *Lonchocarpus campestris* leaves (Mart ex. Benth) have acted differently over the germination of *L. sativa* seeds. The extract of fresh leaves did not affect germination, on the other hand, the extract of dry leaves reduced germination, which was sharper in higher concentrations of 8%. According to Ferreira (2004), changes in germination pattern can result on effects on DNA transcription and translation, membrane permeability, operation of secondary messengers, of breathing (through oxygen sequestration), of enzyme and receptors conformation or the combination of these factors. Allelopathic compounds, since they interfere in cell division, membrane permeability, and enzyme activation, are considered as germination and growth inhibitors (BEN EL HADJ ALI *et al*., 2014). Thus, the reduction in seed germinability shows a cytotoxic effect that may be caused by the allelopathic action of negative influence by the extract on *L. sativa* seeds.

The results of growth biotests are shown in Table 3, expressed through the percentage of growth inhibition in relation to the positive control (plant with distilled water).

Table 3. Growth inhibition percentage (%IG) for *Lactuca sativa cv*. Grands rapids in aqueous extracts of *Psidium cattleianum* Sabine leaves collected in summer and winter.

Leaves	**%IG**	
	WU	**WE**
Summer	57.06 ± 4.85^{aA}	57.96 ± 0.35^{aA}
Winter	51.56 ± 2.44^{aA}	55.33 ± 1.28^{aA}
Control	0.00 ± 0.01^{bA}	0.00 ± 0.01^{bA}

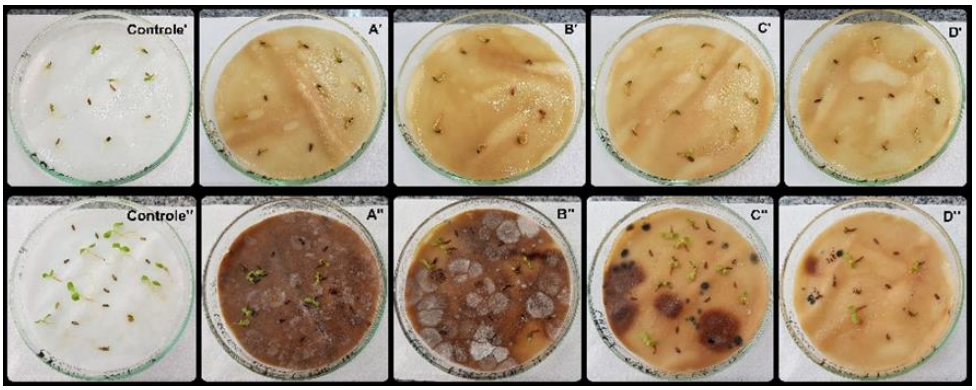
Tukey Test with 95% reliability. Different lowercase letters (in the column) correspond to different experiments ($p<0.05$), when comparing the seasons when the leaves were collected and control. Different uppercase letters (in line), correspond to different experiments ($p<0.05$), when comparing extraction methods. WU and WE correspond to extraction methods using water+ultrasound and water+enzyme, respectively. In the control experiment, distilled water was used for wetting. Source: Prepared by the author, 2019.

For the %IG, when compared WU and WE extracts for a same season (in line), it is verified that, for both extracts, there was no statistical difference ($p>0.05$) among extraction methods, being possible to use both the ultrasound just as the enzyme complex to obtain the extract. When compared WU and WE extracts separately and between seasons (in column), it is verified that, for both extracts, there was no statistical difference ($p>0.05$) between extract of summer leaves and winter ones. Therefore, the results have showed that extracts have exerted inhibition in plant growth of 50%, demonstrating the allelopathic effect of *Psidium cattleianum* Sabine leaves. In many studies, it is observed a lower effect of extracts on germination when compared to initial development, given that the germination process uses reserves of the seed itself (MARASCHIN-SILVA; AQÜILA, 2005). However, it was observed, in this case, that the values were close in both biotests. Reports from other studies point out that the effect commonly caused by extracts on initial growth is the reduction in root sizes (ÁQUILA, 2000), considering that this effect was observed in the treatments performed.

Sausen *et al.* (2009) have also reported this impact in treatments with aqueous extracts of *Acca sellowiana* and *Eugenia involucrat*e leaves. It was also verified, during the experiment with yellow cattley guava, that these effects were followed by changes by morphologic alterations in the roots in the experiment, among which, reduction and lack of growth zone and necrosis. Cruz-Ortega *et al*. (1998) have reported that the stiffening and darkening of radicular apexes are evidence of morphologic and ultra-structural alterations caused by phytotoxins. Thus, it is confirmed that *Psidium cattleianum* Sabine can be used as a potential allelopathic substance, and it must be tested as a possible natural resource in the form of herbicide.

Figure 5 shows the growth biotests for *Lactuca sativa cv*. Grands rapids plantlets treated with WU and WE extracts of winter and summer leaves, collected in the 1st and 5th day of growth.

Figure 5 – Growth biotests of *Lactuca sativa cv.* Grands rapids plantlets treated with WU and WE extracts of winter and summer leaves, collected in the 1st and 5th day of growth



A' and C' correspond to the 1st day of growth in WU extract of summer and winter leaves, respectively. B' and D' correspond to the 1st day of growth in WE extract of summer and winter leaves, respectively. A" and C" correspond to the 5th day of growth in WU extract of summer and winter leaves, respectively. B" and D" correspond to the 5th day of growth in WE extract of summer and winter leaves, respectively. WU and WE correspond to extraction methods using water+ultrasound and water+enzyme, respectively. Source: Prepared by the author, 2019.

It can be observed that the allelopathic influence on lettuce plantlet growth happened in the abnormality specially in the radicular system, in which roots presented necrosis, damages and even lack of roots. The plants radicular system is the most sensitive one to the action of allelochemicals, given that its elongation depends on cell divisions that, if inhibited, compromise its normal development (HOFFMANN *et al.*, 2007) The presence of abnormality in the roots can be considered a good parameter for the registry of plantlet abnormality, since this organ is more sensitive to the allelopathic activity than the aerial part. According to Pires and Oliveira (2001), effects such as darkening and stiffening are secondary effects of allelopathy in response to changes happening at the cell level, an effect observed in many allelopathy studies.

4.4. CONCLUDING REMARKS

The results showed that *Psidium cattleianum* Sabine leaves have shown a content of total phenolic compounds that present antioxidant and allelopathic activity. Generally, the extraction methods used, with the help of ultrasound and enzymes, did not affect the tested activities, however, the season when leaves were collected had influence. In relation to the phenolic content, leaves collected in winter have higher values, while summer leaves had higher antioxidant activity, with IC_{50} of 37.31 ± 0.89 $\mu g.mL^{-1}$ for ultrasound aqueous extraction and 159.63 ± 0.47 $\mu g.mL^{-1}$ for the one with the help of enzymes. Besides that, extracts from all tested samples and extractions showed considerable allelopathic activity, which suggests that leaf extracts can be tested as bioherbicides. The results highlight the significance of this plant, native in the South of Brazil.

5. CHAPTER 3

In this chapter the scientific paper entitled "*Antimicrobial activity of extract of Psidium cattleianum Sabine leaves*" is presented. The article provides an introduction about the subject, the materials and methods used for the development of the research, the results and discussions and the conclusion. The bibliographic references used are presented at the end of the dissertation.

5.1. INTRODUCTION

The *Psidium cattleianum* Sabine (Myrtaceae) is a Brazilian native species that can be found from Bahia to South states, and also in the neighbor country Uruguay (PEREIRA *et al*., 2018a). Commonly known as Cattley guava, is being considered a plant with latent perspectives for the pharmaceutical and food industry due to its potential application as herbal, functional food, among others (PATEL, 2012) most probably due to the presence of bioactive compounds, such as phenolic compounds and carotenoids (MEDINA *et al*., 2011). Species rich in phenolic compounds, ascorbic acid and carotenes are usually associated with prominent biological properties such as increased protection against cellular oxidation, antimicrobial and anticarcinogenic activities (KATALINIĆ *et al*., 2010; PATEL, 2012; RIBEIRO *et al*., 2014).

Besides its fruits being consumed in natura, experiments with extracts from its leaves have demonstrated antiproliferative activity in cancer cells, as well as antioxidant activity in relation to the radicals DPPH, FRAP, and ABTS, and antimicrobial activity for microorganisms *Streptococcus mutans*, *Salmonella* Enteritidis, *Staphylococcus epidermidis*, *Bacillus subtilis* and *Micrococcus luteus* (BIEGELMEYER *et al*., 2011; BRIGHENTI *et al*., 2008; PEREIRA *et al*., 2018b; MEDINA *et al.*, 2011; PATEL, 2012; VERMA *et al*., 2013).

Recently there has been a lot of attention focused on producing medicines and products that are natural, where the search for natural origin products with pharmacological properties has significantly contributed to the discovery of new substances that have important uses. This suggests that plants which manifest relatively high levels of antimicrobial action may be sources of compounds that can be used to inhibit the growth of foodborne pathogens (BISWAS *et al*., 2013; SCUR *et al*., 2016).

Infectious diseases are major causes of death world-wide. Infections with bacteria are associated with high morbidity and mortality especially with immunocompromised patients. The main strategies to prevent and control infectious diseases include public health improvements in hygiene and sanitation, safe water initiatives, as well as the use of antimicrobial agents (SOLIMAN *et al*., 2016). The inappropriate and indiscriminate use of synthetic antimicrobials is leading to the selection of multi-resistant strains, the antimicrobial potential of plant extracts is intended to delay this process through the emergence of new antimicrobial substances (WEBER *et al*., 2014).

Surfaces carry a small risk of direct transmission of infection but may contribute to secondary cross-contamination by hands and instruments or products that may be contaminated by contact with such surfaces and subsequently contaminate persons or other surfaces.

Based on the above, the objective of the present study was to evaluate the antimicrobial potential of aqueous extracts of *P. cattleianum* Sabine against gram-positive and gram-negative bacteria, as well as to test the possibility of using the extracts as surface disinfectants.

5.2. MATERIALS AND METHODS

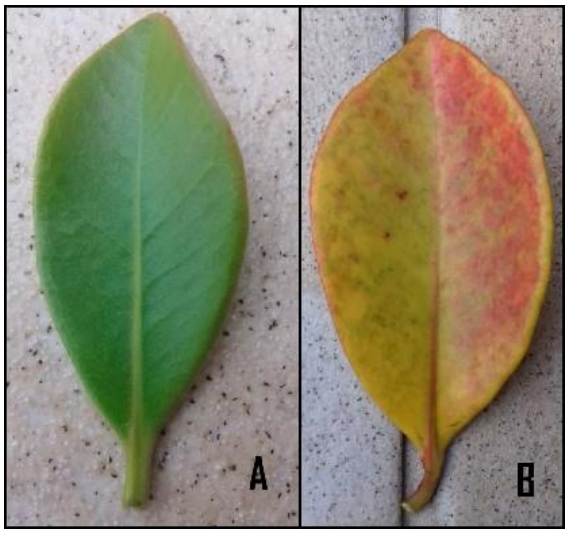
The following are the materials and methods used in the research.

5.2.1. Plant material and sample preparation

Cattley guava leaves (*Psidium cattleianum* Sabine), yellow morphotype, were collected in the southern region of the State of Santa Catarina (28°19'31.9" a 28°19'36.5" S; 49°03'50.3” a 49°03'51.9" W), from July to September 2017 (winter) and from December to March 2018 (summer).

Leaves were selected according to uniform coloration, ruling out vegetal material with rottenness, injuries and/or defects, as shown in the figure 6. The vegetal material was sanitized with gauze dampened with distilled water and dried in a forced circulation stove with air at 40 ±5ºC, to constant mass. After drying, the leaves were manually and separately milled, and sieved in 8 Mesh netting, which finally obtained the samples for extraction.

Figure 6 – Features of leaves of *P. cattleianum* Sabine collected in summer (A) and winter (B).



Source: Prepared by the author, 2019.

5.2.2. Preparation and obtaining the extracts

The procedure to obtain the extracts was adapted from Larrauri *et al*. (1997). For the extraction, 15 g of leaves collected in summer and winter were used, separately, and two method were used of each fraction, WU extraction (water + ultrasound) and WE extraction (water + enzyme), for both seasons, winter and summer, in a total of 4 aqueous extracts.

To obtain WU extracts, 100 mL of distilled water was added to the leaf samples, separately. After that, the mix was taken to a ultrasound bath (70 W) for 3 hours. This mixture stayed at rest, in the dark, for other 3 hours. The supernatant was filtered with quantitative filter paper (‘Whatman’ no. 40), and stocked in a volumetric flask of 100 mL, involved in foil paper. To obtain WE extracts, 100 mL of distilled water was added to leaf samples, separately, and 20 µL of a cellulase complex (Novozymes 22086) was added to the mixture. The solution was taken to a bath with orbital agitation at 100 rpm and 45ºC for 6 hours. The supernatant was filtered with quantitative filter paper (‘Whatman’ no. 40) and stored in a volumetric flask of 100 mL, involved in foil paper.

Right after the extraction, the extracts were stored in Eppendorf tubes and kept frozen at -83 ºC until its use.

5.2.3. Scanning electron microscopy

In order to verify the influence of the extraction method on the structure of leaves, photomicrographs were obtained using scanning electron microscopy (SEM) (microscope Philips, model XL30). The gold coating was carried out in a BAL-TEC Sputter Coater, model SCD 005, for 120s on the dried leaves (leaf samples were previously dried at 30 ºC for 24 hrs).

5.2.4. Antimicrobial activity

The antimicrobial activity of the extracts was evaluated by disc diffusion assay and by determining the minimal inhibitory concentration (MIC), according to Ostrosky *et al*. (2008) and Oliveira *et al.* (2016). The tests were performed against Gran positives bacteria (laboratory stock) *Listeria monocytogenes*, *Staphylococcus aureus* and the Gran negatives (laboratory stock) *Salmonella* Enteritidis and *Escherichia coli.* Pathogenic cultures were recovered in BHI and incubated at 36 °C, overnight. Cultures were left at the concentration of 0.5 of the McFarland scale (equivalent to 10^8 Colony Forming Units per milliliter – $CFU.ml^{-1}$) and diluted in peptone water of casein to the concentration of 10^5 $CFU.ml^{-1}$.

5.2.4.1. Disc diffusion

The microorganisms were inoculated, via swabs, into plates containing Müller-Hinton Agar. Thereafter, three Whatmann filter paper disks sterile of 6 mm diameter were added to each plate. On the paper disks were added 15 µl of the extracts. Sterile distilled water was used for the negative control. Plates were incubated at 35±1 °C for 24 hours. The diameters of the inhibition halos were measured with a pachymeter and the result expressed in millimeters (mm). The higher the inhibition halo, the greater the antimicrobial activity of the extract against the microorganisms tested. All the tests were performed in triplicate.

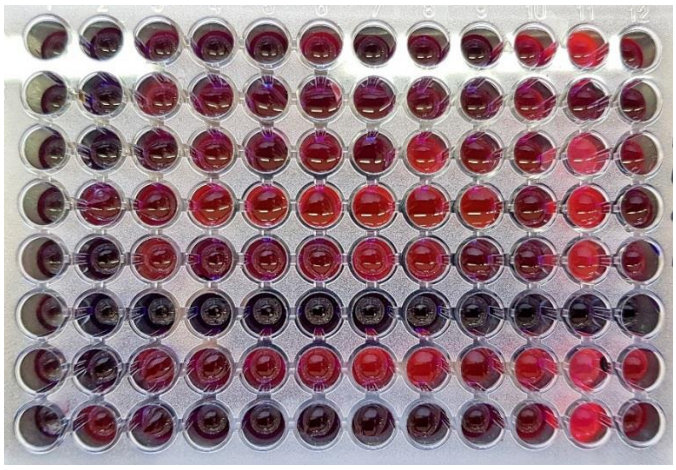
5.2.4.2. Minimum inhibitory concentration (MIC)

The extracts of the leaves of Cattley guava that presented antimicrobial activity through the disc diffusion, were submitted to determination of the MIC, in sterile 96-well plate. Serial dilutions (up to 4 times) of the samples of the extracts were performed in BHI to obtain the desired concentration range. 100µl of a bacterial suspension in BHI was added into each well. Negative control wells (containing the BHI and 200 µl of extract without the bacterial

suspension) and positive control wells (containing BHI and the bacterial suspension) and were also prepared. The plates were incubated at 37°C for 18 hours without shaking. After time, 10 µl of 3% resazurin was added and left for another 2 hours in the incubator at 37 °C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of extract that inhibited the visible growth of the bacteria.

Figure 7 shows the plate used for the MIC, and the change of coloration from blue to red. Where blue indicates absence of microbial growth, while red indicates the presence of viable growing cells.

Figure 7 - Determination of minimum inhibitory concentration (MIC) by plaque microdilution technique.



Source: Prepared by the author, 2019.

5.2.5. Use of plant extracts for surface disinfection

After testing the antimicrobial activity of all the microorganisms, the bacterium *Listeria monocytogenes* was chosen for this test because it presented the worst performance against the tested extracts.

5.2.5.1. Preparation and obtaining the extracts

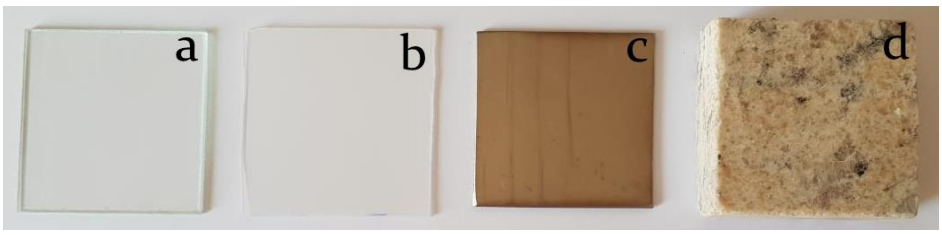
The extracts were obtained as described in item 5.2.2., with only one modification. The extraction cycles were repeated four times for both extracts, WU and WE.

5.2.5.2. Desinfection of surfaces

Four surfaces were used: glass, plastic, stainless steel and granite, with 5 cm2 each (approximately 25 cm2), as shown in Figure 8. The surfaces were contaminated in an aseptic environment with 0.1 mL of suspension of L. monocytogenes. Thirty minutes after contamination the control group (without disinfection) was collected with swabs previously moistened in 0,1% (p/v) peptone water solution, which were transferred to tubes containing 9 mL of 0.1% (p/v) peptone water solution, vortex shaken for 2 min. Serial dilutions was preparad and plated on agar Listeria according to Ottaviani and Agosti (ALOA).

Afterwards, each surface was submitted to the disinfection process by the spray wipe spray technique for each solution, and each surface received the application with the aid of a spray bottle, then it was wiped with sterilized gauze and the disinfectant (extracts), which remained on the surface for 10 minutes. After the disinfection period, samples were collected After the disinfection period, samples were collected as described for the control. The swabs were immersed in 9 mL of sterile saline solution, homogenized in vortex for 2 minutes and inoculed 0,1 mL to agar ALOA. The plates, control and post-disinfection were incubated at 37ºC for 24 hours in a bacteriological oven. After incubation, the Colony Forming Units (log CFU. cm2) were counted (BAMBACE *et al.*, 2003).

Figure 8 – Glass (a), plastic (b), stainless steel (c) and granite (d) surfaces used in desifection tests.



Source: Prepared by the author, 2019.

5.2.6. Statistical analysis

The statistical analysis of experimental results was performed using the software Statistica® 10.0 Statsoft Inc.), performing the Tukey Test, with 95% of reliability.

5.3. RESULTS AND DISCUSSION

Staphylococcus aureus is a major human pathogen that causes a wide range of clinical infections. It is a leading cause of bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections (TONG *et al.*, 2015; WERTHEIM *et al*., 2005). *Listeria monocytogenes* is a relevant foodborne pathogen in public health, responsible for outbreaks of listeriosis often associated to the consumption of ready to eat dairy, meat and fishery products. Listeriosis is a serious disease that can lead to death and mainly affect the elderly, children and immunocompromised individuals (JARVIS *et al.*, 2016; JORDAN; MCAULIFFE, 2018). *Escherichia coli* is a common cause of diarrheagenic illness globally, is the most common cause of uncomplicated and complicated urinary tract infections, and a leading cause of bacteremia and neonatal meningitis (POOLMAN, 2016). *Salmonella* Enteritidis is one of the important sources of salmonellosis cases and outbreaks. These illnesses have been attributed to eggs and egg-containing foods more than any other food (HOWARD *et al*., 2012).

The antimicrobial activities (in mm) of aqueous extracts of *Psidium cattleianum* Sabine leaves collected in summer and winter are shown in Table 4. A classification scheme where the extracts with halos of inhibition <9 mm were classified as inactive; from 9-12 mm is partially active, from 13-18 mm is active and, >18 mm is very active (OLIVEIRA *et al*., 2016).

Table 4 - Antimicrobial activities (in mm) of aqueous extracts of *Psidium cattleianum* Sabine leaves collected in summer and winter.

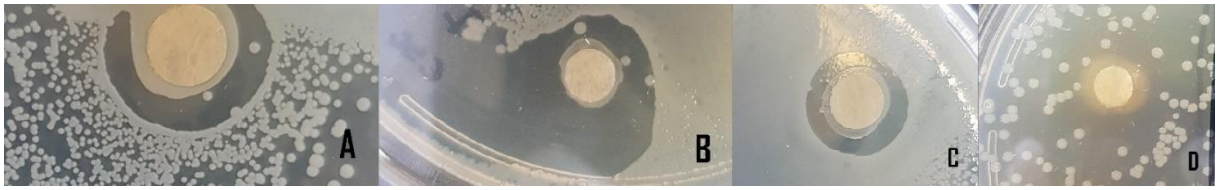
Leaves	**Method of extraction**	**Gram-positive bactéria**		**Gram-negative bacteria**	
		S. aureus	*L. monocytogenes*	*E. coli*	*S.* Enteritidis
Summer	WU	12.52 ± 1.86aA	18.03 ± 1.05aA	9.49 ± 0.00aA	11.20 ± 0.00aA
	WE	18.21 ± 0.00aA	23.12 ± 0.08aB	15.22 ±0.09aB	13.92 ± 0.00aB
Winter	WU	10.67 ± 1.01aA	18.54 ± 1.11aA	15.36 ± 0.09bA	12.73 ± 1.00aA
	WE	17.18 ± 2.03aB	18.71 ± 1.16bA	17.04 ± 1.08aA	17.46 ± 0.08bB

Zone of Inhibiton was determined by agar disc diffusion. Results were presented as means ± standard deviation. WU and WE correspond to extraction methods using water+ultrasound and water+enzyme, respectively. Different lowercase letters correspond to different experiments ($p<0.05$), when comparing the seasons when the leaves were collected, for the same extraction method. Different uppercase letters correspond to different experiments ($p<0.05$), when comparing extraction methods, for the same season. Source: Prepared by the author, 2019.

For the tested bacteria (Gram-positive and Gram-negative), aqueous extracts of *Psidium cattleianum* Sabine leaves that showed antimicrobial activity can be classified as partially active to very active, that is, none of the extracts tested was classified as inactive. In general, it was observed that Gram-positive bacteria presented lower resistance against the extracts tested when compared to Gram-negative bactéria. This fact may be related to the structure of the bacterial cell, since the Gram-positive bacteria present a single layer in the cell wall, whereas the Gram-negative bacteria present an extra layer of lipopolysaccharides and proteins in the cell wall that form a barrier of permeability to antimicrobial agents (FORSYTHE, 2013).

Figure 9 shows the best zones of Inhibiton for all tested microorganisms.

Figure 9 – Zones of Inhibiton that was determined by agar disc diffusion, for all tested microorganisms.



A corresponds to the best zone of inhibition against *S. aureus*, *B* the best zone of inhibition against *L. monocytogenes*, C the best zone of inhibition against *E. coli* and D the best zone of inhibition against *S.* Enteritis. Source: Prepared by the author, 2019.

Regarding the season, the WE extract was statistically different ($p < 0.05$) when compared to the two microorganisms *S.* Enteritidis and *L. monocytogenes*; whereas for the WU extract only *E. coli* showed a statistically different in relation to the two seasons. For *S. aureus*, independent of the collection season, there was no statistical difference in relation to WU and WE extracts.

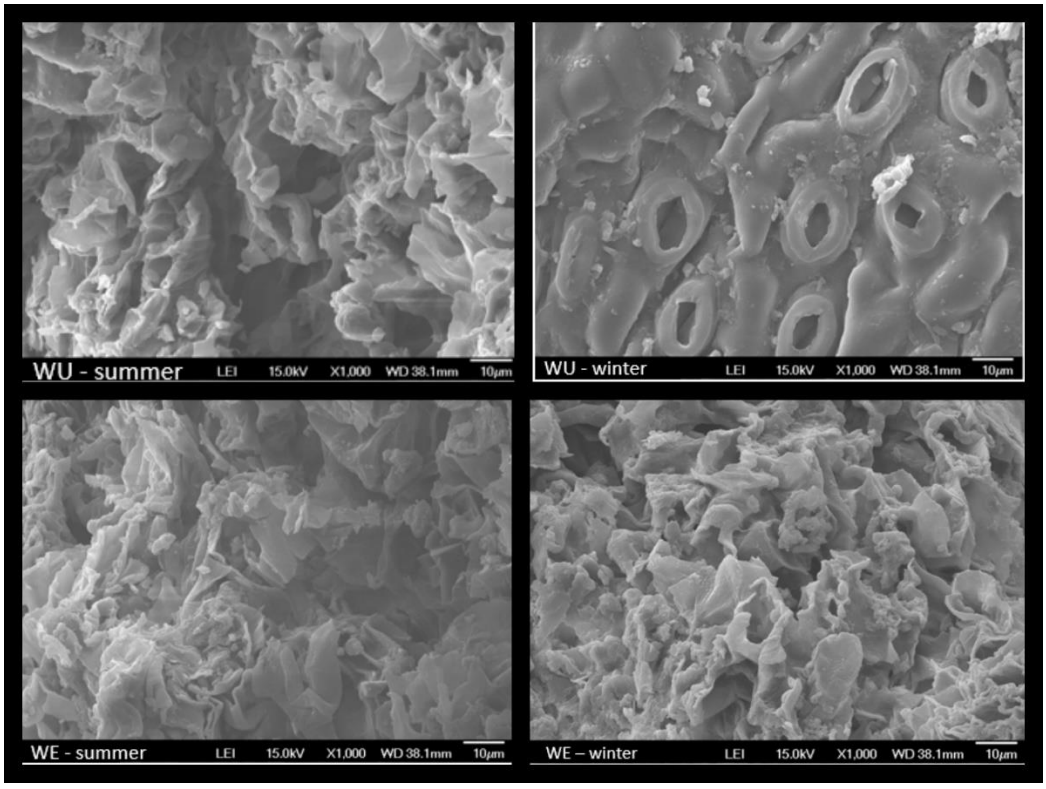
Gobbo-Neto and Lopes (2007) highlight that seasonal variation on the contentes of almost all classes of secondary metabolites, such as phenolic acids, flavonoids, coumarins, and saponins. Annual, monthly, and even daily temperature variations are one of the factors exerting higher influence of the development of each species, affecting, thus, the production of secondary metabolites.

When comparing the extracts WU and WE for the same season, it can be verified that the results varied according to the microorganism used. For *S.* Enteritidis the results showed a significant difference ($p < 0.05$), independent of the season, which shows that the extraction mode affected the content of the compounds responsible for the antimicrobial activity. For *E.*

coli and *L. monocytogenes*, the results presented significant difference, only in the summer season. While for *S. aureus* the results presented significant difference only during the winter season.

Figure 10 shows the SEM of the leaves submitted to extraction using ultrasound and the enzyme complex .

Figure 10 - SEM of the leaves submitted to extraction using ultrasound (WU – summer and WU – winter) and the enzyme complex (WE – Summer and WE – winter).



Source: Prepared by the author, 2019.

It is possible to observe that there was a difference between the extracts WU and WE, which shows that the extraction form affected the cell structure of the leaves. For the extraction of the WU, it was verified the formation of cavities in the vegetal structure of the leaves, which possibly happened due to the phenomenon of cavitation. Cavitation causes the formation of cavities, where the dissolved gases in the system migrate, forming microbubbles, which increase and decrease in size, generating cycles of expansion and compression until the bubbles implode, releasing large amounts of heat and exerting high pressures close to (CARCEL *et al.*,

2012; VEILLET *et al*., 2010). The presence of solid materials in the system causes an asymmetrical implosion of the microbubbles, generating jets that collide with the solid surfaces. These collisions cause plant cells to be disrupted, facilitating the diffusion of the solvent extracts into the matrix (DE CASTRO; CAPOTE, 2010; SHIRSATH et al., 2012). For the extraction of WE, the occurrence of hydrolysis of the plant structure. Plants comprise primary and secondary cell walls, both of which are fortified by cellulose microfibrils. Primary cell walls typically contain cellulose, hemicellulose (xyloglucans), pectin and proteins. Secondary cell walls are composed of cellulose, hemicellulose and lignin and constitute the majority of cell wall mass (VOGEL, 2008). Therefore, mixtures of enzymes and complexes with multiple activity are more efficient than the isolated enzymes (JORDAN *et al*., 2012). The enzyme complex used is composed of cellulases, which are enzymes classified as glycosyl hydrolases and that degrade the plant cell wall. Three enzymes are part of this group, they are called endoglucanases, exoglucanases and beta-glycosidases. Endoglucanases act in the inner region of the cellulose fiber and release smaller compounds formed by a few glucose units, the so-called oligosaccharides (small sugars). Exoglucanases act at the ends of the cellulose fibers and release units of free glucose or cellobiose, which are smaller compounds, formed by two glucose units. Beta-glycosidases break the chemical bond existing between the two glucose units that form the cellobiose, releasing (free) glucose units (CASTRO; PEREIRA, 2010; OZIOKO *et al*., 2013).

Alvarenga *et al*. (2016) have assessed the chromatographic profile of yellow cattley guava leaves and observed the prevalence of compounds such as quercetin, quercitrin, and isoquercitrin, all part of the flavonoid class, and ellagic acid, as well as lower amounts of catechin, chlorogenic acid and kaempferol. The delineation of the possible mechanism of action of flavonoids is hampered by conflicting findings. It is known that the flavonoids act in bacterial cells through the formation of complexes between proteins and the cell wall, causing its rupture (TAGURI *et al*., 2004). The flavonoids lacking hydroxyl groups on their b-rings are more active against microorganisms than are those with the 2OH groups; this finding supports the idea that their microbial target is the membrane. Lipophilic compounds would be more disruptive of this structure. However, several authors have also found the opposite effect; i.e., the more hydroxylation, the greater the antimicrobial activity. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. However, lipophilic flavonoids may also disrupt microbial membranes (COWAN, 1999; SATO *et al*., 1996).

On the other hand, tannins act in the microorganisms by preventing their growth through the inhibition of the transport of nutrients and the formation of complexes between the tannins and the bacterial cell wall (MCSWEENEY *et al.,* 2001). Finally, the action mechanism of triterpenoids in microorganisms is associated with the disruption of lipophilic compounds of microbial membranes, causing their death (TEPE *et al*., 2004).

The antibacterial activity of aqueous extracts of *Psidium cattleianum* Sabine leaves expressed as minimum inhibitory concentrations (MICs) are shown in Table 5. The antibacterial activity of plant extracts can be considered significant when MIC values are lower than 100 μg/ml, moderate when 100 <MIC ≤ 625 $\mu g.mL^{-1}$ and low when MIC>625 $\mu g.mL^{-1}$ (KUETE, 2010; TCHINDA *et al.,* 2017). The MIC values ranged from 6.28 $\mu g.mL^{-1}$ to 35.95 $\mu g.mL^{-1}$ and are considered significant values. Therefore, the obtained MIC values are very important when considering importance of the tested bacterias.

Table 5 - Antibacterial activity of aqueous extracts of *Psidium cattleianum* Sabine leaves expressed as minimum inhibitory concentrations (MICs), in $\mu g.mL^{-1}$.

Leaves	**Method of extraction**	**Gram-positive bacteria**		**Gram-negative bacteria**	
		Staphylococcus aureus	*Listeria monocytogenes*	*Escherichia coli*	*Salmonella* enteritidis
Summer	WU	12.57	12.57	6.28	12.57
	WE	15.09	15.09	30.19	7.55
Winter	WU	17.97	17.97	35.95	17.97
	WE	15.37	15.37	30.75	15.37

WU and WE correspond to extraction methods using water+ultrasound and water+enzyme, respectively. Source: Prepared by the author, 2019.

Scur *et al*. (2016) when investigating the phytochemical screening and antimicrobial activitie of extracts of *Psidium cattleianum* Sabine reported that for the aqueous extract the MIC ranged from 6.25 to 50 $mg.mL^{-1}$ for Gram-negative bacteria and 6.25 to 12.5 $mg.mL^{-1}$ for Gram-positive bacteria, which shows that the result was three times higher than that stated in the literature.

Regarding the disinfection of surfaces, all the extracts tested presented significant positive results against *Listeria monocytogenes* on the four surfaces tested, as can be shown in the Table 6.

Table 6 – Disinfection power (logCFU.cm^{-2}) of aqueous extracts of *Psidium cattleianum* Sabine leaves against *Listeria monocytogenes* in glass, plastic, stainless steel and granite surfaces.

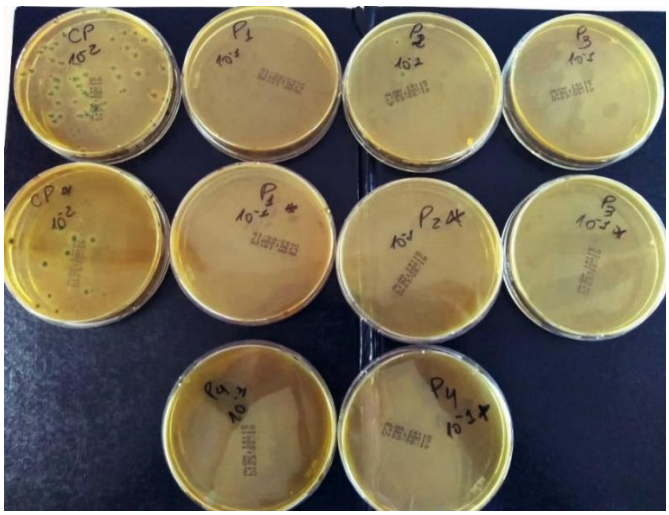
Leaves	**Method of extraction**	***Listeria monocytogenes***			
		Surfaces			
		Glass	Plastic	Stainless steel	Granite
Summer	WU	0.00	0.00	0.00	0.00
	WE	0.00	0.60	0.00	0.00
Winter	WU	0.00	0.00	0.00	0.00
	WE	0.00	0.00	0.00	0.00
Control		1.38	3.09	3.25	1.78

WU and WE correspond to extraction methods using water+ultrasound and water+enzyme, respectively. Source: Prepared by the author, 2019.

All extracts tested inhibited the growth of *L. monocytogenes* as compared to the control sample. Only the WE - summer extract did not present null count against the tested plastic surface. However, as the control sample presented values of 3.09 (logCFU.cm^{-2}), it was observed that it was efficient in the reduction of 2.49 (logCFU.cm^{-2}) of the count.

The figure bellow shows the desinfection power of aqueous extracts of Psidium cattleianum Sabine leaves against plastic surface.

Figure 11 – Control plate (CP), winter leaves extract and enzyme (P1), summer leaves extract and enzyme (P2), summer leaves extract and US (P3) and winter leaves extract and US (P4) used in desifection tests.



Source: Prepared by the author, 2019.

It is known that the chemical composition of the surface influences bacterial adhesion and proliferation. In materials with different functional groups in their chemical composition, there is a difference in the number of cells adhered due to the fact that the bacteria-surface interaction depends on the hydrophobicity and loading of the material (KATSIKOGIANNI; MISSIRLIS, 2004).

Silva *et al*. (2008) when studying the adhesion to and viability of *L. monocytogenes* on food contact surfaces reported that the results show different patterns of adhered cell viability for the different surfaces. The results also demonstrated that the percentage of viable cells on the surface of glass and polypropylene was close to 100%, despite the lower extent of adhesion. Cells adhered to granite exhibited reduced viability despite the high number of adhered cells.

Beresford *et al*. (2001), investigating the adhesion of *Listeria monocytogenes* to seventeen different materials approved for food use, representing metals, rubbers and polymers, reported surprise that there was no difference in the degree of fixation that occurred instantaneously or after 2 hours. Mafu *et al*. (1990) demonstrated that the binding of *L. monocytogenes* cells to stainless steel, glass, polypropylene and rubber surfaces did not show an increasing trend with longer contact times (20 min versus 1 hrs), but the presence of extracellular materials around cells attached to surfaces.

The adhesion of *L. monocytogenes* to abiotic surfaces is a phenomenon dependent on several factors, which demonstrates the importance of a better understanding of microbial viability, since adherent bacteria that remain viable are the true culprits for postprocess contamination , as well as the search for new disinfectant agents that can derail the adhered cells.

5.4. CONCLUNDING REMARKS

The results showed that the leaves of *Psidium cattleianum* Sabine present considerable antimicrobial activity. Generally, the extraction methods used, with the help of ultrasound and enzymes and the season when leaves were collected did affect the tested activity. In relation to the zone of inhibition, the best results were found against *L. monocytogenes* bacteria, with inhibition halos measuring up to 23 mm. For the minimum inhibitory concentrations (MICs), all the extracts tested showed significant activity, with values lower than 100 μg/ml. Regarding

the disinfection of the surfaces, all the tested extracts presented positive results against *L. monocytogenes* on the four surfaces tested. The results highlight the significance of this plant, native in the South of Brazil.

6. FINAL CONSIDERATIONS

The extracts obtained from the leaves of *Psidium cattleianum* Sabine had the presence of phenolic compounds, regardless of the method of extraction used, either with ultrasound or with the aid of enzymes, but the content of these differed in relation to the season of collection of the leaves. The extracts showed the ability to remove free radicals considerably when the DPPH radical was used, as well as showed ability to inhibit bacterial growth for both Gram-negative and Gram-positive bacteria of interest to the food industry. Regarding the disinfection of surfaces, all the extracts tested presented significant positive results against *Listeria monocytogenes* on the four surfaces tested. They also demonstrated allelopathic activity against *Lactuca sativa*, indicating their possible use as a bioherbicide.

In general, the results of the present study may serve as a basis for the use of aqueous extracts of *Psidium cattleianum* Sabine in the treatment of diseases caused by oxidative and infectious stress, through the development of new antioxidant agents, antibiotics, and may indicate a possibility for use as a natural herbicide.

The use of other radicals for the determination of antioxidant capacity, the chromatography and identification of the phenolic compounds present in the extracts, the inhibition capacity of other bacteria as well as fungi and yeasts important for the food industry are suggested for future work, pharmaceutical and environmental area. Also, tests of allelopathy with other indicator plants existent in the specialized literature and the performance of toxicological and cytotoxic tests are suggested.

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