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Assessment in Vitro of Antibacterial Activity of Manipulated Product, on Solution Form, Obtained from Dry Extract of Leaves of *Psidium guajava L.*

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Abstract

The *Psidium guajava L*. specie is a perennial shrub, belonging to the *Myrtaceae* family and it is popularly known as guava, its leaves are used in therapy for treating various diseases. The study aims to evaluate the antibacterial activity *in vitro* of manipulated product obtained from dried extract of the leaves of *P. guajava* L. front standard bacteria ATCC and clinical isolates. The tests were conducted on bacterial samples: *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27883), *Escherichia coli* (ATCC 25922), *Salmonella spp*, *Acinetobacter baumannii*, *Proteus mirabilis*, *Shigella flexneri*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Streptococcus agalactiae*, *Streptococcus mutans*. Among the tests can be mentioned phytochemical of the *P. guajava* leaves ethanolic extract (EE), microbiological control and physical-chemical analysis of the product and microbiological tests such as agar diffusion method (wells), minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), an evaluation test of hemolytic capacity of the solution and in vitro assay cytotoxic activity were performed, . The best result of the product in the agar diffusion method was front *Staphylococcus epidermidis*, while the lower MIC and MBC were front *Staphylococcus aureus* (ATCC 25923). The product showed no hemolytic activity and no cytotoxic activity at the tested concentrations. According to the test results, it is believed on the possibility of the production of a pharmaceutical formulation derived from the dry extract of *Psidium guajava*, since it showed great antibacterial activity.

Keywords: *Psidium guajava*, Antibacterial activity, Ethanolic extract

1. Introduction

Because of the biodiversity present in different biomes in Brazil, there is growing demand for natural products by domestic and international pharmaceutical industries, which drives scientific research for natural drugs. A study can be more effective if the investigation cover the pharmacological potential of various species of a particular genus guided by popular medicinal use (Duarte, 2006).

According to the World Health Organization, medicinal plant it is "all and any plant which has in one or more organs, substances that can be used for therapeutic purposes or are precursors semisynthetic drugs" (World Health Organization [Who], 1998). It has been proved the importance of medicinal plants to the population, especially populations with low purchasing power. These populations found simple and easy solutions due the tradition of using medicinal plants to various diseases (Vieira & Ara újo, 2012). The big problem in this practice is the lack of direction for much of the population that uses this type of treatment, so that there is in some cases an aggravation of the problem, poisoning or drug interactions (Filho, 2009).

Due to the great accessibility and use of medicinal plants therapy in Brazil and worldwide, the standardization of the control of drugs is necessary. The plants need to be properly cultivated, collected, identified, must be free of foreign matter, parts of other plants and inorganic or microbial contamination. It is necessary that medicinal plants meet certain quality standards in order to meet the minimum criteria of efficiency and safety (Souza-Moreira, 2010).

According to Cunico (2004) due to bacterial resistance, which has occurred over the decades since the creation of

penicillin, the treatment options for infectious processes are becoming smaller and smaller. Members of the family Enterobacteriaceae, *Pseudomonas*, and *Streptococcus* are examples of antibiotics resistant organisms old one of the outputs which might be taken to limit bacterial resistance would be improved or creation of antimicrobial agents (Neu, 1992).

The World Health Organization report of Health on global surveillance of microbial resistance shows that resistance is no longer a forecast for the future, this is happening at the present time, around the world, and it is endangering the ability to treat common infections communities and hospitals. Without urgency or coordinated action, the world is heading towards a post-antibiotic era, in which common infections, which have been treatable for decades, may kill again (Who, 2014).

Due to the problem of bacterial resistance to antibiotics available on the market, it was necessary to further research on the study and evaluation of natural products with antimicrobial property. Among the main tools in this context, it highlights ethnopharmacological studies (Albuquerque & Hanazaki, 2006). Natural products have shown to be quite effective from the standpoint of antimicrobial activity (Cunico, 2004; Maia, 2009; Aresi, 2011).

Studies of Souza et al. (2007) primarily responsible for antimicrobial activity of medicinal plants can be flavonoids and tannins. According to Schenkel et al. (2002) among the secondary metabolites produced by plants, saponins are one of the most prominent classes because of its wide distribution in the plant kingdom and their important biological activities as well as anti-inflammatory, antimicrobial activity, hemolytic and antiviral.

Saponins are compounds originating from the secondary metabolism of plants, usually found in the most vulnerable tissues to fungal attack, bacterial or predatory insects. These compounds act as a chemical barrier or as a protector of the plant's defense system (Lima, 2009). The saponin is a very present metabolite in *Psidium guajava* (Maia, 2009).

Native to tropical America and is now distributed in all tropical and subtropical regions of the world, including in some temperate regions of Europe in altitude up to 1,200 m. There are over 90 varieties of guava, and most production is in Brazil, India, Colombia, Cuba, the United States and Mexico (Maia, 2009).

The species *P. guajava* L. has several therapeutic indications and is grown around the Brazil and the world. The use of guava plant parts are signals of the importance of scientific research in the same, in order to evaluate its effects on therapies, as well as validate its use of standards for the rational use of medicinal plants. This study differs to present a comparative approach between the action of the extract from the leaves with pharmaceutical product from the same plant.

Thus, this work is done in order to determine the antimicrobial activity of the extract of guava leaves (*Psidium guajava* L.) and product derived from dried extract of the leaves of this plant against a selection of microorganisms of clinical interest and ATCC standard strains. This may open our possibility of treatment front some bacteria.

2. Material Studied

2.1 Type of Study and Place of Research

This work is a descriptive experimental study in Microbiology Laboratory of Pharmacy building at UFMA (Federal University of Maranhão.

2.2 Botanical Material

P. guajava L. used to prepare the EE is cataloged and identified in the Herbarium Atico Seabra UFMA under the number 1203. This botanical material was collected in São Luís / MA at UFMA.

2.3 Ethanolic Extract Obtaining

The ethanolic extract (EE) of the *P. guajava L*. (guava) leaves were obtained from the fresh plant by cold maceration process. Thus, it was obtained 0.1 g / ml concentration of the ethanolic extract from the plant 50g and 450 ml of ethanol (99.5%). Initially, the leaves were duly selected, dried, ground and packaged in glass with screw cap and protected from light with aluminium foil for 14 days with the extractor solvent being agitated periodically. Thereafter, it was filtered, and the ethanol extract obtained, and packed in suitable container amber glass. The dry weight was obtained 292,5g. The preparation of the extract was held at the Pharmacy Microbiology Laboratory of the Federal University of Maranhão, where it also was performed the tests of antibacterial activity.

2.4 Manipulated Product: Solution

A product has been manipulated from the dried extract: a hydroalcoholic solution (Pharmacopoeia, 2010). The alcohol solution was prepared with 20% alcohol, glycerin, propylene glycol and water (qs) in addition to put 1% of the dry extract of leaves of *P. guajava* L., placed in an amber vial. A quantity of basic product was separated for use as controls in the tests with the product (hydroalcoholic solution).

3. Results and Discussion

3.1 Phytochemical Analysis of EE of P. guajava Leaves

Based on the results obtained and the comparison with the data described in the literature, it is clear the presence of secondary metabolites of *P. guajava* (Ilha, 2008; Okamoto, 2010). The results are classified as negative (-), weak positive (+), moderate positive (++) and strong positive (+++). Phytochemical analysis of EE showed the presence of flavones, flavonoids and xanthones, as well as condensed tannins and saponins classified as moderately positive, as shown in Table 1.

Table 1. Phytochemical screening of the ethanol extract of *P. guajava* leaves

Secondary Metabolites	Results [1]
Phenols	-
Hydrolysable tannins	-
condensed tannins	++
Flavones, flavonols and xanthones	++
Chaconas and auronas	-
Leucoantociacianidinas, catechins	-
Steroids	-
Triterpenoids -	-
Alkaloids	-
Coumarins	-
Saponins	++

[1] + ++ strongly positive, ++ moderate positive, + positive, -negative

Source: Prepared by the author (2015).

Flavonoids are bioactive compounds that come from vegetable source present in the humans diet and exhibit many biological properties. As example of the flavonoid activities, they have the ability to modulate many enzymes, and action on the vascular system including anti-inflammatory action. Furthermore, they act in reduction of atherosclerotic plaques, inhibition of platelet aggregation, vasodilation promotion, hormonal action (especially isoflavones) and significant antioxidant activity (Dovichi; Lajolo, 2011).

Most vegetables carriers tannin, which may be in several parts of the plant as the roots, wood, bark, leaves, fruits, seeds and sap. However, tannin content varies not only from a plant to another as a part to another of the same plant (Battestin *et al*., 2004).

Tannins have various applications mainly relating astringent properties. Among the functions that it is intended we can highlight the antidiarrheal effect, antiseptic, antimicrobial (Ilha *et al*., 2008) and antifungal (Monteiro *et al*., 2005). Furthermore, the tannins are haemostatic and may serve as an antidote in cases of poisoning. In healing process of wounds, burns, and inflammation, tannins assist forming a protective layer on the epithelial tissues injured and may just below this layer, the healing process naturally occurring (Monteiro et al., 2005).

Saponins are found in fruits, vegetables, nuts, seeds, stems, flowers, tea, wine, honey and propolis. They form a foam (like soap) when they are mixed and stirred in an aqueous medium. The antimicrobial activity of saponins was proven when extracted from the roots, stem bark, leaves and wood of certain plants (Food Brazil, 2010).

3.2 Analysis of the Manipulated Product

The obtained product had a good primary stability. No phase separation occurring or another instability in the tests performed as centrifugation. As regards the sensory characteristics presented solution is dark brown probably due to the color of the extract of *P. guajava* leaves. Likewise it can characterize the odor characteristic of the plant remained although other substances making up the product.

3.3 Chemical and Physical Analysis and Microbiological Control of the Product

The results of microbiological control and chemical and physical analysis of the product (solution 1%) is described in Table 2 and 3, respectively. The results of the microbiological control indicates that no contamination of the solution, since the results showed no presence of microorganism in all three culture media used since the presumptive evidence to the confirmatory test.

Table 2. Microbiological Control Result of the Solution

Microbiological Control		
Parameter	Culture Medium Used	Results
heterotrophic bacteria	Lauryl broth	Absent
Total coliforms	Brilliant green broth	Absent
Thermotolerant coliforms	EC broth	Absent

Source: Prepared by the author (2015).

The chemical and physical characteristics of the solution were analyzed to establish the primary stability analysis of the product. In this analysis the formulation is presented within the appropriate standards. The pH of skin is around 4,9 to 5.9 and the pH of the solution was 5.1 indicating the possibility of a product of topical use.

Table 3. Result of Chemistry and Physics Analysis of the Solution

Chemistry and Physics Analysis		
pH	Density	Viscosity
5.1	34.6521g	4380 mPa-s

Source: Prepared by the author (2015).

3.4 Screening: Diffusion Method of the EE and Solution in Agar

The antimicrobial activity of EE was noted in solid culture medium front some bacterial samples. The biggest halo obtained from EE that remained close in the use of the solution was front S. epidermidis. As noted in Table 4 bacterial samples that had higher halo to the solution were: *A. baumannii*, *S. flexneri*, *S. mutans*, *S. haemolyticus* and *S. epidermidis*. As a negative control, we used the base product and the solvent from the ethanol extract, ethyl alcohol 99.5%. As a positive control was used tobramycin (10mcg).

Table 4. Inhibitory activity of ethanol extract of *P. guajava* and the solution front clinical isolates and reference strains of bacteria through the agar diffusion test

Microrganisms	Inhibition zone (mm)		
	Extract[a]	**Product**[b]	**NC**[c]
ATCC *Staphylococcus aureus*	19	13	0
ATCC *Pseudomonas aeruginosa*	14	12	0
ATCC *Escherichia coli*	10	10	0
Acinetobacter baumannii	16	15	0
Proteus mirabilis	20	12	0
Salmonella spp	13	14	0
Shigella flexneri	20	17	0
Streptococcus agalactiae	15	14	0
Streptococcus mutans	16	15	0
Staphylococcus epidermdidis	23	19	0
Staphylococcus haemolyticus	18	15	0

a Ethanolic extract of P. guajava leaves (0.1 mg/ml), b Solução obtained from dried extract of the leaves of P. guajava (0.01 mg / mL), c Negative Control – Product base.

Source: Prepared by the author (2015)

3.5 Minimum Inhibitory Concentration (MIC) of EE and Solution

Concerning the EE, the lowest MIC found was 3.125 $mg.ml^{-1}$ for samples of ATCC *S. aureus*, *A. baumannii* and *S. epidermidis*. For bacteria *S. agalactiae*, ATCC *P. aeruginosa* and ATCC *E. coli*, the MIC found was 6.25 $mg.ml^{-1}$. In the case of the solution, the better results observed of the MIC were front ATCC *S. aureus* with 0.156 $mg.ml^{-1}$, *A. baumannii* 0.625 $mg.ml^{-1}$ and *S. epidermidis* with 1.25 $mg.ml^{-1}$.

Future studies are needed to determine the MIC of isolated active ingredients found in the leaves of *P. guajava* since the action of active ingredients in isolation may be present in much lower concentrations. The results of MIC were determined as shown in Table 5.

Table 5. Minimum inhibitory concentration in vitro of *P. guajava* ethanolic extract and solution front clinical isolates and reference strains of bacteria

Microrganism	Minimum inhibitory concentration (mg/ml)		
	Extract[1]	**Solution**[2]	**NC**[3]
ATCC *Escherichia coli*	6,250	2,5	0
ATCC *Staphylococcus aureus*	3,125	0,156	0
ATCC *Psedomonas aeruginosa*	6,250	2,5	0
Acinetobacter baumannii	3,125	0,625	0
Streptococcus agalactiae	6,250	5	0
Staphylococcus epidermdidis	3,125	1,25	0

[a] Ethanolic extract of *P. guajava* leaves (0.1 mg/ml), [b] Solução obtained from dried extract of the leaves of *P. guajava* (0.01 mg / mL), [c] Negative Control – Product base.

Source: Prepared by the author (2015)

The EE of *P. guajava* leaves have a weak inhibition compared with the solution to all tested bacterial samples, probably because the crude extract. Regarding the solution was a good antibacterial activity front the bacteria tested among these gram-positive and gram-negative bacteria. However, it is emphasized that there was better activity front gram-positive

bacteria at a lower concentration, as in standard ATCC *S. aureus* bacteria. Other authors also found the antimicrobial activity of *P. guajava* extract (Gonçalves, 2008; Okamoto, 2010)

The efficacy of a medicinal plant may not be due to a major active component, but the combined action of different compounds originally in the plant. There are studies in the literature about the synergistic effect between extracts with antibiotics where there is an increased clinical efficacy of these antibiotics by the extracts, such as extract of *P. guajava*, for example (Maia *et al*., 2009). This fact demonstrates the importance of future studies in order to select a synergistic blend of assets with improved therapeutic properties.

The antibacterial activity of plant extract front to *Acinetobacter baumannii* has been reported by Borges (2007) being flavonoids possibly responsible for the activity, it is also mentioned by Lucarini (2009). Another metabolite that may be responsible for antibacterial activity are tannins (Scalbert, 1991) and saponins (Food Brazil, 2010).

3.6 Minimum Bactericidal Concentration (MBC) of the EE and Solution

The best results analyzing MBC of the EE of *P. guajava* leaves was 1.56 $mg.ml^{-1}$ in the sample of *S. epidermidis* and 3.125 $mg.ml^{-1}$ compared to standard sample (ATCC) *S. aureus* as shown in table 6. For the solution the best MBC was presented front standard bacteria ATCC *S. aureus* being 0.156 $mg.ml^{-1}$, after this result highlights the MBC for clinical isolates of *A. baumannii* and *S. epidermidis* being 1.25 $mg.mL^{-1}$.

Table 6. Minimum bactericidal concentration of the ethanolic extract of *P. guajava* leaves and solution front reference strains and clinical isolates bacteria

Microrganism	Minimum bactericidal concentration **(mg/ml)**		
	Extract[1]	**Solution[2]**	**NC[3]**
ATCC *Staphylococcus aureus*	3.125	0.156	0
ATCC *Psedomonas aeruginosa*	12.5	5	0
ATCC *Escherichia coli*	12.5	2.5	0
Acinetobacter baumannii	6.25	1.25	0
Streptococcus agalactiae	6.25	0	0
Staphylococcus epidermdidis	1.56	1,25	0

[a] Ethanolic extract of *P. guajava* leaves (0.1 mg/ml), [b] Solução obtained from dried extract of the leaves of *P. guajava* (0.01 mg / mL), [c] Negative Control – Product base.

Source: Prepared by the author (2015)

It shows the importance of this product as a possible therapeutic option, since these bacteria are highly prevalent among multidrug-resistant bacteria found in hospitals. A study of Andrade (2006) showed that between multidrug-resistant bacteria found in ICU, 19% were *Staphylococcus aureus* and 14.3% were *A. baumannii*.

The nosocominais infections, which are mainly caused by *S. epidermidis* and *S. aureus*, are a serious problem in hospitals also occurring with greater occurrence in intensive care units (ICUs) (Michelim et al, 2005). The labor of Michelim et al. (2005), there was 76.5% of 98 clinical isolates of *S. epidermidis*, analyzed in ICU patients, which were demonstrated as multiresistant to antibiotics.

3.7 Search Hemolytic Activity in the Culture Supernatant (Microplate)

In this study, the sheep and horse blood hemolysis test in vitro were employed to evaluate the hemolytic activity of the derivative solution of the dry extract of the leaves of *P. guajava*.

The highest concentration of the solution used was 5 and 0.3125 $mg.mL^{-1}$ was the lowest concentration in the test. Under these conditions was not observed positive result. It means that the derived product from dry extract has no hemolytic activity at the concentrations tested. In addition, this test is indicative of cytotoxic activity, and this resulted in a good premise in this regard, however it is necessary to carry out further tests.

3.8 In Vitro Assay Cytotoxic Activity

Cytotoxicity is expressed as the concentration of the substance inhibiting cell growth by 50% (CD50). Three replicate plates were used to determine the cytotoxicity of each sample, and this test showed that the samples used there were no cytotoxic activity. This resulted in a good premise in this regard; however, it is necessary to carry out further tests.

4. Methods

4.1 Phytochemical study of EE of P. Guajava Leaves

Qualitative phytochemical screening was performed according to Matos (2009). This test has the purpose of detecting the presence of classes of secondary metabolites. To identify the steroids and triterpenes group was used the reaction of Lieabermann-Buchard; the class of phenols and tannins applied the reaction with an alcohol solution of ferric chloride;

classes for anthocyanins, proanthocyanidins, flavonoids, leocoantocianianidinas, catechins, flavonols, flavonones and xanthones has been used the pH change and Dragendorff test, Mayer and Hager for alkaloids.

To the reaction with ferric chloride was added 3 to 5 drops of 1% ferric chloride aqueous solution in 1 mL of aqueous extract. The development of blue color occurs in the presence of tannins; green coloring in the presence of flavonoids; and brown color in the presence of poly phenol. The methodology employed for determination of polyphenols and total tannin content is based on the ability of the phenolic compounds react with metal salts, as in the case of FeCl3 in alkaline medium, forming blackish coloring solutions and the ability of tannins to precipitate compounds protein product, the complexation of phenolic hydroxyl groups with the amine of amino acids (Couto, et al, 2009).

The reaction Libermann Bouchard consists on evaporate 30 mL of EE in a water bath until dry. Dissolve the residue in 5 ml of chloroform and filtered. With the aid of a pipette, it was taken from each of the fractions, the following quantities three different test tubes: 0.1 ml; 0.5 mL; 1.0 ml. Then the volume was completed to 2 ml with chloroform. In Chapel, was added 1 ml of acetic anhydride and 2 ml of sulfuric acid (H_2SO_4) concentrated. The change of the color of the extract to pink or blue staining indicates the presence of steroid or triterpenes with carbonyl function (C = O) on carbon 3 and double bond between carbons 5 and 6 of the structure. Being this change of color with the greatest occurrence, although there may be changes to green color, which indicates hydroxyl function (OH) on the carbon 3 and double bond between carbons 5 and 6. The yellow color indicating possible methylation (CH 3) on carbon 14 (Machado, Nakashima, Silva, & Krüger, 2011). In this method consisting in treating the sample in the presence of acetic acid and a few drops of sulfuric acid, there is dehydration and oxidation of cyclopentanoperhydrophenanthrene ring system forming an aromatic steroid, which is evidenced by the appearance of a blue-green color (Queiroz, 2009).

It was transferred 2 ml of EE for five test tubes and proceeded the alkaloids research by adding 2 drops of the following reagents: reactive Mayer (tetraiodide mercury potassium) and Dragendorff reagent (tetraiodide potassium bismuth). It was observed if there was a white precipitate formation or light white turbidity for the reactive Mayer and brick color precipitate for Dragendorff reactive. The tests are described below in Table 7 with their goals and principles.

Table 7. Metabolites classes analyzed by phytochemical screening of the extract of *P. guajava* leaves

Secondary Metabolites	**Tests**
Phenols	Ferric chloride test
Hydrolysable tannins	Ferric chloride test
Condensed tannins	Ferric chloride test
Anthocyanidin, anthocyanin and flavonoids	pH change Test (pH 3; 8.5 e 11)
Flavones, flavonols and xanthones	pH change Test (pH 3; 8.5 e 11)
Chaconas and auronas	pH change Test (pH 3; 8.5 e 11)
Leucoantociacianidinas, catechins	pH change Test (pH 3; 8.5 e 11)
Steroids	Libermann-Bouchard test
Triterpenoids	Libermann-Bouchard test
Alkaloids	Dragendorff, Mayer e Hager test

Source: Prepared by the author (2015)

4.2 Microbiological, Chemical and Physical Control of the Product

The preliminary stability test was performed, and this test was conducted by centrifuge, viscosity, pH, sensory characteristics (color, odor and appearance) and density (Brazilian Pharmacopoeia, 2010).

To determine the pH was used digital pH meter, evaluating the potential difference between two electrodes immersed in the sample. The electrode was inserted directly into the solution (Brazilian Pharmacopoeia, 2010). The specific density was determined in pycnometer, coupled with thermometer, previously weighed empty. The sample was inserted into the pycnometer and the temperature was adjusted to 20 °C, whereupon the pycnometer was weighed again. The difference between the mass of the pycnometer with the sample and empty pycnometer is the mass of the sample. The ratio between the sample mass and the mass of water, both at 20 °C represents the specific density of the test sample (Brazilian Pharmacopoeia, 2010). The viscosity was measured at Brookfield viscosimeter, which measures the viscosity of a pharmaceutical form by the force required to rotate the spindle in the liquid being tested (Brazilian Pharmacopoeia, 2010). The test using the centrifugation was used as primary stability parameter (Brazilian Pharmacopoeia, 2010).

The sample was analyzed in order to ascertain gross features that indicate instability. The stability is indicated by the non-occurrence of phase separation, precipitation and turbidity. The odor was examined by smell and color was examined by visual comparison under white light conditions.

The microbiological control of the product was done with three races being one a presumptive test, in which it was inoculated 1 ml of the solution in a series of 3 tubes containing broth sodium lauryl sulfate. The tubes were then incubated at 36 °C for 24 to 48 hours. A confirmatory test of total coliform was made from the peal of each positive tube lauryl for the tubes with bright green bile broth 2% lactose and incubated at 36 °C for 24 to 48 hours. The

confirmatory test for fecal coliform was taken from each subculture positive bright green tube to tube with EC broth and incubated at 45 °C for 24 to 48 h. After the period determined for each test observations were carried out (Brazilian Pharmacopoeia, 2010).

4.3 Strains: Bacterial

Bacterial samples are from the Clinical Microbiology Laboratory at Federal University of Maranhão. The tests were performed using standard micro-organisms (ATCC - American Type Culture Collection) and clinical isolates samples. Bacterial samples are: *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27883) and *Escherichia coli* (ATCC 25922). The clinical isolates samples are: *Salmonella, Acinetobacter baumannii, Proteus mirabilis, Shigella flexneri, Staphylococcus epidermidis, Staphylococcus haemolyticus, Streptococcus mutans* and *Streptococcus agalactiae.*

4.4 Preparation of Bacterial Suspensions

The microorganisms were initially reactivated from their original cultures and maintained in liquid BHI medium (Brain Heart Infusion) at 37 °C for 24h. Subsequently, the samples were grown on Mueller Hinton plates for 37 °C for 18-24 hours. Isolated colonies were then resuspended in 3 ml of saline (0.9% NaCl) sterile until turbidity equivalent in the range of 0.5 McFarland (1.5 x 108 CFU / mL).

4.5 Screening: Diffusion method in EE Agar and Solution

To carry out this test the following aforementioned bacterial samples in item 4.3 were used.

The antimicrobial potential of hydroalcoholic solution and EE was initially evaluated by diffusion technique in Mueller Hinton. The wells were identified and 30 uL of the solution and EE were added with the pipet (Clsi, 2012). As a positive control was used tobramycin (10mcg) and as a negative control ethanol 99.5% and the product base. Then the material was taken to the incubator at 35 °C for 24 hours. The following day, the formed halos were measured in millimeters with a ruler. The results of the screening method of diffusion in agar was obtained from the average of triplicate results.

After screening test for all other tests the following samples were used: *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27883), *E. coli* (ATCC 25922), *A. baumannii. S. epidermidis and S. agalactiae.*

4.6 Minimum Inhibitory Concentrations (MIC) of the EE and Solution

The MIC determination was performed using the macrodilution method (Phillips, 1991; Piddock, 1990; Nccls, 2003; Clsi, 2007). For this test each bacterial suspension was homogenized in BHI broth in a ratio of 1: 1000 (v / v), which was obtained from a bacterial concentration of about 1-2 x 105 CFU / mL. Sterile glass vials were prepared with 5 ml of serial dilutions sterile on ratio of 2 of EE and product. Also, 100 ul solution of 2,3,5-triphenyltetrazolium chloride (CTT) at 1% dilution were added in each tube whose bacterial growth makes it red solution. After dilution, 30μL of each inoculum was transferred to tubes. The negative control to EE was 99.5% ethanol and the product base to the solution. The tubes were then homogenized in with the aid of a vortex at low speed, and incubated under the same conditions described above. The MIC was the lowest concentration of EE and the solution where there is no bacterial growth. The CIM was determined after the triplicate experiment.

4.7 Minimum Bactericidal Concentrations (MBC) of the EE and Solution

The tubes incubated for determination of MIC in the liquid medium were used to determine the MBC (Phillips, 1991; Piddock, 1990; Clsi, 2007). An aliquot (1ml) of each of it was inoculated into Mueller Hinton Agar plates, and subsequently these plates were incubated at room at 37 °C for 24h. The MBC have been considered for the lowest concentration of the EE or solution where there is no bacterial growth on the surface of the inoculated agar (99.9% of microbial death). The MBC was determined after the triplicate experiment.

4.8 Search of Haemolytic Activity in the Culture Supernatant (Microplate)

The hemolytic activity was assayed by incubating the extract with 1% erythrocytes (sheep and horse) washed 3 times with PBS (phosphate buffer saline), pH 7.2 for 1 hour at 37 °C at the bottom of ELISA 96-well plates. The product was added in 5 wells varying the average from 5 to 0.156 $mg.ml^{-1}$ against all erythrocytes. The well control was added to the base product for the solution. As positive control, the water was used, and as negative control a buffer solution was used. In addition, held the same procedure to the controls (Bhakdi *et al*., 1986). The hemolytic activity was expressed as the maximum concentration of extract and products that do not cause hemolysis. The hemolytic activity was determined after the triplicate experiment.

4.9 Cell Culture

HeLa cell lines (human cervical carcinoma), A-549 (human lung carcinoma), HT-29 (human colon adenocarcinoma) and Vero (monkey kidney) were obtained from the American Type Culture Collection (ATCC). The cells were grown in

RPMI 1640 supplemented with 10% fetal calf serum 1% (w/v) glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B. Cells were cultured in a humidified atmosphere at 37 ºC in 5% CO2. The macrophages were isolated from mice and kept according to the methodology described by Tseng et al. (2006).

4.10 In Vitro Assay Cytotoxic Activity

Cells were washed with phosphate-buffered saline (PBS) free of magnesium and calcium. After PBS decantation, cells were detached by addition of 0.025% trypsin-EDTA and PBS to a final volume of 50 ml and centrifuged. The pellet was suspended in 10 ml of a medium to obtain a single cell suspension. The density of viable cells was determined by Trypan blue exclusion in a hemocytometer and the preparation was diluted with a medium to yield previously determined optimal plating densities for cells. Before the assay, 5 x 104 cells / well were seeded on 96-well plates and the suspension was incubated 24 h at 37 ℃ to cell attachment. After 24 h, the cells were treated with the essential oil and terpenes. The oil was dissolved in ethanol and a serial of doubling essential oil dilution was added to five replicate wells, over the range of 600 - 0.6 µg/ml against all cell lines and macrophages. Terpenes were also dissolved in ethanol and tested to five replicate wells, but over the range of 200 – 0.2 µg/ml. The final concentration of ethanol in the culture medium was kept at 0.5% (v/v) to avoid solvent toxicity. The activities of the essential oil and terpenes were considered according to the survival of 50% or less cells after an exposure time of 72 h. The cell culture used as control received only 0.5 % ethanol at final concentration. Cytotoxicity was measured using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After an exposure time of 72 h, the medium was removed and then, MTT assays were performed using the cell titer kit (Promega Corp., USA). On a 96-well plate, 20 ml of MTT (5 mg/ml) in PBS was incubated with cells for 2 h at 37 ℃. After this period, the medium containing MTT was removed and 100 ml of acidified isopropanol (0.04 mol/l HCl) was added. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad Laboratories, model 3550, USA). Cell viability was expressed with respect to the absorbance of the control wells, which were considered as 100% of absorbance. Cytotoxicity is expressed as the concentration of the substance (essential oil and terpenes) inhibiting cell growth by 50% (CD50). Three replicate plates were used to determine the cytotoxicity of each sample (Stavri *et al*., 2005; Hou *et al*., 2006, Xiao *et al*., 2006).

4.11 Statistical Analysis

Data are reported as the mean ± SD for at least three replicates. Statistical analysis was performed using the Student t-test, with significance level set at $P < 0.05$.

5. Conclusion

The study of the solution and EE of *Psidium guajava L*. leaves allowed to evaluate the antibacterial activity in relation to different bacterial samples tested both gram-negative and gram-positive.

Conducting a comparative study of the activity of an EE of *P. guajava L*. leaves with a product derived of dry extract from the same plant as opens up the possibility for innovation in the pharmaceutical sector with the possibility of new antibacterial pharmaceutical product.

The lowest minimum inhibitory concentration (MIC) was found for the hydro-alcoholic solution was 0.156 $mg.ml^{-1}$ front ATCC *S. aureus* and lower minimum bactericidal concentration was found to be 0.156 $mg.ml^{-1}$ front the same bacterium. It is noteworthy that the best results of the product are given front bacteria usually prevalent mutirresistentes in hospital.

The tests indicated that the product formulation based on natural extracts is viable way, and that the extract incorporated into a formulation may still be responsible for improving its activity with regard to its antibacterial properties.

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