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Phenolic composition and medicinal usage of *Psidium guajava* Linn.: Antifungal activity or inhibition of virulence?

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Abstract *Psidium guajava* is a Myrtaceae plant whose medicinal properties are recognized in several locations. The use of teas and tinctures prepared from their leaves has been used to combat infections caused by fungi of the genus *Candida*. In this study, aqueous extracts of leaves and hydroethanolic were tested to verify the antifungal potential and its chemical composition has been investigated. The microbiological assays were performed by broth microdilution to determine the minimum inhibitory concentration (MIC) and from these the minimum fungicidal concentration was performed (MFC) by subculturing on solid media. A cell viability curve was obtained for demonstration of inhibition of fungal growth of strains of *Candida albicans* and *Candida tropicalis*. Tests to check morphological changes by the action of the extracts were performed in microcultive cameras depleted environment at concentrations of MIC/2, MIC and MIC × 2. Extracts analyzed by high performance liquid chromatography demonstrated flavonoids and phenolic acids. The extracts showed fungistatic effect and no fungicide with MIC > 8192 μg/mL, MFC above 8192 μg/mL. The IC_{50} was calculated ranging from 1803.02 to 5623.41 μg/mL. It has been found

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that the extracts affect the morphological transition capability, preventing the formation of pseudo-hyphae and hyphae. Teas and tinctures, therefore, have the potential antifungal, by direct contact, causing inhibition of fungal multiplication and its virulence factor, the cell dimorphism, preventing tissue invasion. Further studies are needed to elucidate the biochemical pathways and genes assets involved in these processes.

1. Introduction

Microorganisms of the genus *Candida* can be found naturally composing the microbiota of the human organism inhabiting your gastrointestinal tract and mucous membranes (Lu et al., 2014; Shao et al., 2007). Changes in dynamic of the host organism too favor the proliferation of these fungi and the disturbance caused in homeostasis can lead to a range of infections that range in their level and location and can only be superficial, in skin and mucosal (oral, vaginal candidiasis) or systemic, compromising the life of an individual (Mayer et al., 2013; Sardi et al., 2013).

Usually the infections caused by *Candida* spp. in its magnitude are assigned to the species *Candida albicans*, however, illness caused by *Candida* non-albicans (CNAM) had increased incidence over the years and yeasts of *Candida glabrata*, *Candida tropicalis*, *Candida krusei* and *Candida parapsilosis* have been increasingly identified as human pathogens (Sardi et al., 2013; Silva et al., 2012).

Mechanisms of resistance to commercial drugs developed by these microorganisms have been constantly investigated and reported and the continuous evolution for resistance is extremely worrying considering the limited number of antifungal classes currently available (Maubon et al., 2014; Xie et al., 2014). The search for different therapeutic alternatives is a constant and the use of natural products of plant origin often serves as a reference to the search for active compounds and, in this sense, a ethno directed approach has directed pharmaceutical research (Albuquerque and Hanazaki, 2006), in this case, in order to antifungal discovery potential.

Member of the Myrtaceae, *Psidium guajava* Linn. species (guava), plant native to tropical America (Okamoto et al., 2009), has its widespread medicinal use among the world's populations. Being a plant of tropical and subtropical regions, can be found on plantations, in backyards of homes, or naturally in other areas, and could even be considered invasive species (Richardson and Rejmánek, 2011).

The medicinal attributes from all parts of the species are spread over several generations and therefore, make up many lists of ethnobotanical studies, showing great versatility and value in use, being mentioned for the treatment of various types of diseases (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008).

This therapeutic use recorded in different locations includes a significant number of body systems such as disorders of the sensory system: vertigo (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008); disorders of the respiratory system: laryngitis, sore throat, colds, coughs, tuberculosis, lung problems, bronchitis, catarrh, rhinitis (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008; Ogbole and Ajaiyeoba, 2010; Waruruai et al., 2011); disorders of the genito-urinary system: menstrual disorders, vaginal discharge, childbirth, nephritis, premenstrual syndrome, gonorrhea, non-specified venereal diseases, leucorrhea (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008; Van Vuuren and Naidoo, 2010); disorders of the nervous system: anorexia, epilepsy, cerebral ailments, chorea, convulsions, nervousness (Dakappa-Shruthi et al., 2013; Gómez-Estrada et al., 2011; Gutiérrez et al., 2008); disorders of the digestive system: diarrhea, dysentery, stomachache, digestive problems, gastric insufficiency, ulcers, dyspepsia, gastroenteritis, gastritis, bowel disorders, colic, toothache, constipation (Dakappa-Shruthi et al., 2013; Gómez-Estrada et al., 2011; Gutiérrez et al., 2008); disorders of the circulatory system: piles, swelling, hypertension, edema (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008); the musculoskeletal system and connective tissue diseases: gout, spasm, rheumatic pain (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008); not defined conditions or pain not defined: aches (Dakappa-Shruthi et al., 2013); skin diseases and tissue subcutaneous: inflamed mucous membranes, mouth – swelling, skin problems, ulcers, itch, scabies, skin sores, wounds, dermatosis, sores, boil, gingivitis (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008); diseases of the endocrine glands, nutrition and metabolism: diabetes (Gutiérrez et al., 2008); infectious and parasitic diseases: cholera, worms, bacterial infections, herpes, mycoses, thrush, pox, measles (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008; Waruruai et al., 2011); neoplasms: cancer (Alonso-Castro et al., 2011), and disease of the blood and blood-forming organs: hemorrhages, blood cleansing (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008).

The *P. guajava* is popularly used in the treatment of infectious diseases, particularly against those caused by fungi, it is common practice registered in different countries such as Brazil, Cuba and South Africa where it is used to treat thrush, leucorrhoea, and vaginitis, pathologies associated with infections caused by *Candida* spp. (Borba and Macedo, 2006; Fenner et al., 2006; Oliveira et al., 2010; Ramírez et al., 2007; Van Vuuren and Naidoo, 2010).

Considering the pharmacological potential of the species *P. guajava* described in ethnobotanical reports, especially with regard to its therapeutic use in treatments against diseases caused by fungi, this study aims to scientifically validate the antifungal properties of tea and tincture prepared with leaves of guava and evaluate the effect of natural products in virulence strains of *C. albicans* and *C. tropicalis*, particularly its morphological transition process.

2. Material and methods

2.1. Collection area

The collection was realized in the rainy season at the county of Milagres, Ceará, Northeastern region of Brail (07° 17.119′ S

and 038° 51.779′ W, 388 m of altitude; 07° 17.120′ S and 038° 51.778′ W, 389 m of altitude; 07° 17.122′ S and 038° 51.776′ W, 392 m of altitude; 07° 17.119′ S and 038° 51.779′ W, 388 m of altitude) at "Sítio" Malhada. The climate is semi-arid, with temperatures ranging between 24° and 26 °C (IPECE, 2013).

2.2. Plant material

The study was conducted using young, healthy leaves of a *Psidium* species locally known as red guava, which were collected and transported to the Laboratory of Microbiology and Molecular Biology at the Regional University of Cariri – URCA. Twigs with flowers of the species were also collected and vouchers were produced and deposited in the Herbarium Dárdano de Andrade Lima at the university under No. 10935, where the species was identified as *Psidium guajava* Linn. The collection period included January, February, March and April, known as the "wintry block of the Cariri Ceara region." Collections were made between 8:30 and 10:30 am, and the plant material was taken to the laboratory where it was subjected to qualitative screening and cleaning before being weighed and stored under refrigeration. Altogether, there was 2650 g of leaves in perfect condition, and this quantity was divided for preparation of three types of extracts: Aqueous Extract of *P. guajava* Infusion (AEPGI), Aqueous Extract of *P. guajava* Decoction (AEPGD) and: Hydroethanolic Extract of *P. guajava* (HEPG).

2.3. Preparation of extracts

2.3.1. Aqueous extracts

Two types of aqueous extracts were prepared, each using 399.9 g of leaves mixed with 6 L of water (based on a proportion of 10 g/150 mL, equivalent to one cup of tea – 150 cc). The decoction was made by mixing roughly cut leaves in cold water and then boiling for 15 min. Afterward, the tea was allowed to cool, filtered and then stored under refrigeration. As for the infusion, the water was boiled without leaves, which were placed in the water after turning off the heat. The pot was covered with a lid and allowed to stand until the tea cooled down (Matos, 2002), and the preparation was then filtered and stored under refrigeration and both infusion and decoction were frozen (−60 °C) and lyophilized to dryness. The powdered extracts were stored under refrigeration for testing, using 14.46 g (yield 3.62%) and 15 g (yield 3.75%) extract powder from the decoction and infusion, respectively.

2.3.2. Hydroethanolic extract

The hydroethanolic extract (70%) was prepared by trituration with cold extraction, using a total of 1846.5 g leaves in a proportion of 5 g/mL of hydroethanolic solution (Matos, 2002). The leaves were cut to increase contact surface with the solvent, and the mixture was left at room temperature protected from air and light, for a period of 96 h for maximum extraction efficiency. The mixture was then filtered and placed in a rotary evaporator (Q-344B – Quimis – Brazil) at 40 rpm and 60 °C to concentrate the extract. Finally, the crude extract was frozen, lyophilized (50.8 g – yield 2.75%) and then stored under refrigeration.

2.4. Chemical analysis

2.4.1. Chemical, apparatus and general procedures

All chemicals were of analytical grade. Methanol, acetic acid, gallic acid, caffeic acid and chlorogenic acid were purchased from Merck (Darmstadt, Germany). Quercetin, quercitrin, isoquercitrin, rutin, kaempferol, luteolin, catechin and epicatechin were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC–DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

2.4.2. High performance liquid chromatography with diode array detection (HPLC–DAD)

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% (B) for 2 min; 25% (B) until 10 min; 40, 50, 60, 70 and 80% (B) every 10 min; following the method described by Silva et al. (2014) with slight modifications. *P. guajava* extracts (hydroethanolic – EHEPG, infusion – EAIPG and decoction – EADPG) and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use, the extracts of *P. guajava* were analyzed at a concentration of 20 mg/mL. The flow rate was 0.6 mL/min and the injection volume was 50 μL. The sample and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standard references were prepared in water: methanol (1:1; v/v) at a concentration range of 0.025–0.300 mg/mL catechin, epicatechin, quercetin, quercitrin, isoquercitrin, kaempferol, luteolin and rutin, and 0.035–0.300 mg/mL for gallic, caffeic and chlorogenic acids. Quantification was carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid, 281 nm for catechin and epicatechin, 327 nm for chlorogenic and caffeic acids, and 366 for quercetin, quercitrin, isoquercitrin, luteolin, kaempferol and rutin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–500 nm). Calibration curve for gallic acid: $Y = 12673x + 1281.9$ ($r = 0.9998$); caffeic acid: $Y = 12943x + 1191.7$ ($r = 0.9996$); chlorogenic acid: $Y = 12083x + 1327.9$ ($r = 0.9995$); catechin: $Y = 11734x + 1306.8$ ($r = 0.9999$); epicatechin: $Y = 12387x + 1239.1$ ($r = 0.9997$); rutin: $Y = 13752x + 1186.5$ ($r = 0.9991$); quercetin: $Y = 11970x + 1181.7$ ($r = 0.9996$); quercitrin: $Y = 11679x + 1251.7$ ($r = 0.9998$), isoquercitrin: $Y = 13759x + 1251.9$ ($r = 0.9993$), kaempferol: $Y = 12659x + 1172.3$ ($r = 0.9997$) and luteolin: $Y = 12507x + 1341.8$ ($r = 0.9990$). All chromatography operations were carried out at ambient temperature and in triplicate.

2.4.3. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent

analytical curves, as defined by Kamdem et al. (2013). LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.5. Antifungal assay

2.5.1. Strains and culture media used

Standard types of strains were obtained from the Culture Collection of Oswaldo Cruz of the Brazilian Institute of Quality Control in Health (INCQS) and clinical isolates of the yeasts *C. albicans* and *C. tropicalis* were provided by Dr. Edeltrudes Oliveira Lima (Mycology Laboratory of Paraíba Federal University), namely CA INCQS 40006, CA LM 62, CA LM 77, CA LM 109, CA LM 111, CA LM 122, CT INCQS 40042, CT LM 18, CT LM 20 and CT LM 23. These strains were inoculated into Sabouraud Dextrose Agar (SDA, KASVI) and incubated for 24 h at 37 °C. Afterward, small aliquots of yeast were transferred to test tubes each containing 3 mL of sterile saline (0.9%). Using the McFarland scale, the concentration of the inoculum was standardized by comparing its turbidity with the 0.5 standard, giving a standard yeast suspension of 1×10^5 cells/mL (NCCLS, 2002). The inocula thus prepared were used to determine the minimum inhibitory concentration (MIC) in Sabouraud Dextrose Broth (SDB, HIMEDIA), double concentrated. Another culture medium was used for analysis of yeast micromorphology. The potato dextrose agar (PDA, DIFCO) was prepared by diluting it more than that recommended by the manufacturer to make it a depleted medium capable of stimulating yeast to produce hyphae. Agar was added to this diluted medium to obtain a solid medium.

2.5.2. Drugs, reagents and preparation of solutions

Dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) was used for dilution of the extracts, and the antifungal fluconazole (Capsule – FLUCOMED), diluted in water, was used as the reference drug. The matrix solutions of the extracts were prepared by weighing 0.3 g of each extract and then diluting in 1 mL of DMSO. To obtain the desired concentration for testing, the extracts were further diluted in sterile distilled water so that the concentration of DMSO in the natural product did not exert any activity in the test cells (Stoppa et al., 2009).

2.5.3. Microbiological screening

Microbiological screening was performed to select the yeasts to be used in microbiological testing. The microdilution broth was chosen to perform this procedure, and this was done by determining the MIC (Javadpour et al., 1996), since the plates prepared to carry out this test would be used later in tests to find the minimum fungicidal concentration, besides facilitating the demonstration of cell viability curve and calculating the IC_{50} of the test products.

2.5.4. Determination of minimum inhibitory concentration (MIC)

This test was performed by the broth microdilution method in 96-well plates. Each well was filled with 100 µL of SDB containing 10% fungal inoculum, and then, 100 µL of the natural product (16,384 µg/mL) or fluconazole (antifungal reference) at the same concentration, was added to the first well, followed by twofold serial dilution. The concentrations in the wells ranged from 64 to 8192 µg/mL. The last well contained no extract or drug and served as the normal growth control (Javadpour et al., 1996). Controls for diluent of the products (using saline instead of inoculum) and the sterile medium were also prepared. All tests were performed in triplicate. The plates were incubated at 37 °C for 24 h and afterward read in an ELISA spectrophotometer (Thermoplate®) at a wavelength of 630 nm. The MIC was defined as "the lower concentration of na antimicrobial agent that inhibit the visible growth of
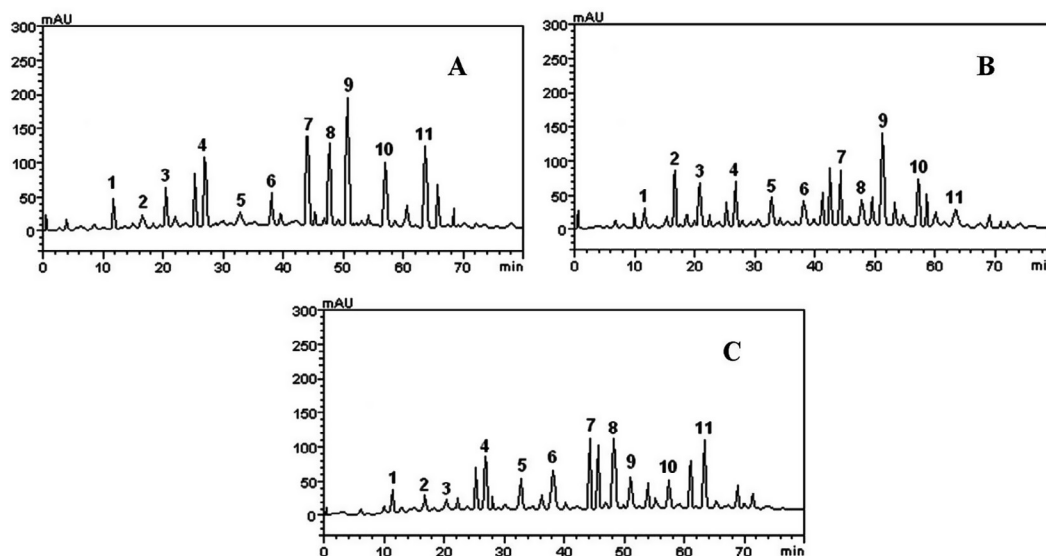

Figure 1 Chromatogram of *Psidium guajava* extracts. High performance liquid chromatography phenolics and flavonoids profile of *Psidium guajava*. (A) Hydroethanolic Extract of *P. guajava* (HEPG); (B) Aqueous Extract of *P. guajava* Decoction (AEPGD); Aqueous Extract of *P. guajava* Infusion (AEPGI); gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), epicatechin (peak 5), rutin (peak 6), quercitrin (peak 7), isoquercitrin (peak 8), quercetin (peak 9), kaempferol (peak 10) and luteolin (peak 11).

Table 1 Phenolics and flavonoid composition of *Psidium guajava*.

Compounds	*Psidium guajava*			LOD µg/mL	LOQ µg/mL
	EHPG mg/g	AEPGD mg/g	AEPGI mg/g		
Gallic acid	3.46 ± 0.01 a	1.57 ± 0.02 a	1.54 ± 0.01 a	0.019	0.062
Catechin	1.57 ± 0.03 b	4.94 ± 0.01 b	1.29 ± 0.02 b	0.008	0.025
Chlorogenic acid	4.39 ± 0.01 c	4.23 ± 0.03 c	1.25 ± 0.03 b	0.024	0.081
Caffeic acid	8.01 ± 0.02 d	4.30 ± 0.01 c	4.73 ± 0.01 c	0.035	0.116
Epicatechin	1.58 ± 0.01 b	3.61 ± 0.02 d	3.58 ± 0.02 d	0.010	0.034
Rutin	3.62 ± 0.01 a	3.52 ± 0.01 d	3.97 ± 0.01 e	0.017	0.056
Quercitrin	11.17 ± 0.03 f	4.95 ± 0.01 b	8.62 ± 0.01 f	0.032	0.105
Isoquercitrin	10.35 ± 0.01 e	3.48 ± 0.03 d	8.59 ± 0.03 f	0.009	0.031
Quercetin	16.81 ± 0.02 g	10.15 ± 0.03 e	3.45 ± 0.03 d	0.025	0.083
Kaempferol	8.26 ± 0.03 d	4.32 ± 0.01 c	3.42 ± 0.01 d	0.018	0.059
Luteolin	10.13 ± 0.01 e	1.69 ± 0.03 a	8.51 ± 0.02 f	0.023	0.075

Results are expressed as mean ± S.E. of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.01$. HEPG: Hydroethanolic Extract of *P. guajava*; AEPGD: Aqueous Extract of *P. guajava* Decoction; AEPGI: Aqueous Extract of *P. guajava* Infusion; LOD: limit of detection; LOQ: limit of quantification.

Table 2 IC_{50} (µg/mL) of *Psidium guajava* extracts against *Candida* strains.

Product tested	*Candida albicans*		*Candida tropicalis*	
	INCQS 40006	LM 77	INCQS 40042	LM 23
AEPGI	3235.94	3890.45	4570.88	4570.88
AEPGD	4797.33	5623.41	5495.41	5128.61
HEPG	1803.02	1905.46	1862.09	1905.46
Fluconazole	76.72	69.09	73.98	58.63

AEPGI: Aqueous Extract of *P. guajava* Infusion; AEPGD: Aqueous Extract of *P. guajava* Decoction; HEPG: Hydroethanolic Extract of *P. guajava*; INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia.

na microorganism in dilution assays" (CLSI, 2002). The results obtained in the ELISA readout were used to construct the cell viability curve and the IC_{50} of the extracts of *P. guajava*.

2.5.5. Determination of minimum fungicidal concentration (CFM)

For this test, a small sterile rod was placed in each well of the MIC test plate (except for sterility control). After mixing the medium in each well, the rod was taken to a large petri dish containing SDA, streaking its surface and transferring the solution (medium + inoculum + natural product) for subculture of yeast and checking cell viability. After 24 h incubation, the plates were inspected for any formation of colonies of *Candida* (Ernst et al., 1999, with modifications). The concentration at which there was no growth of fungal colonies was considered the MFC of the natural product.

2.5.6. Effect of natural products on fungal morphology

To determine if the natural product caused any change in fungal morphology, by inhibiting the development of hyphae, sterile micromorphological chamber slides were prepared for observation of yeasts. Three milliliters of PDA medium depleted by dilution were added to chambers, containing the natural product concentrations MIC/2, MIC and MIC × 2. Aliquots of the inoculi were taken from the petri dishes to make two parallel steaks on the solid medium, which were then covered with a sterile coverslip. The chambers were placed in the incubator for 24 h (37 °C) and inspected under a light microscope using a 40× objective. A camera was attached to the microscope to capture images. A control for yeast growth (hyphae stimulated by depleting medium) was performed, as well as a control with the conventional antifungal fluconazole for comparative purposes and a control with DMSO at 100% and 0.5% (the concentration in the natural products used in the tests). The assays were performed according to Sidrin and Rocha (2010) and Mendes (2011), with some modifications.

2.6. Statistical analysis

The results of the tests were done in triplicate. Data obtained for each sample and concentration were checked for their normal distribution and then analyzed by one-way ANOVA by post hoc Tukey test. EC_{50} values were obtained by nonlinear regression for the purpose of interpolating values from standard curves (using the software Graphpad Prism, v. 5.0) of the % *growth* values plotted against concentration and EC_{50} values are expressed as µg/mL.

3. Results and discussion

The chemical analysis was performed to detect the presence of phenolic acids and flavonoids, verifying the predominance of the latter in two extracts. The hydroethanolic extract showed more efficient as extracting agent, since quantity of compounds able to extract higher. To the aqueous extracts, exposure to different temperature times caused no significant difference in the amount of extracted compounds, however the extract obtained from infusion had a greater abundance of flavonoids as compared to the decoction. Studies show that can occur degradation of flavonoids with temperature rise, however, this

process also depends on the chemical structure and the interaction between them (Baby et al., 2007; Mello et al., 2010). In this sense, the decoction longer exposure to elevated temperature may have been the cause of the reduction of the level of flavonoids. The major compound differed only in the analysis of each extract into the aqueous infusion made, which in this case is the quercitrin, while the other the quercitrin appears to be the most expressive content. The chromatogram extracts of the species are shown in Fig. 1, and the results representing their chromatographic profiles in front the parameters used are detailed in Table 1.

The chemical composition of *P. guajava* has been widely investigated and studies have reported that plant extracts are constituted alkaloids, triterpenoids, tannins, saponins, glycosides, flavonoids, and phenolic compounds and other compounds (Dakappa-Shruthi et al., 2013; Tambe et al., 2014).

A chemical analysis of tea from the leaves of guava (Chang et al., 2013) highlighted a polyphenol profile in which the main components were found: quercetin, myricetins, catechin, gallic and ellagic acids and their derivatives, but the researchers call attention to differences in chemical composition, stating that several factors such as time of collection, form of collection and processing, temperature, among others, may influence the outcome of the chemical prospecting.

The data obtained in microdilution test were used for MIC determination, assembly of cell viability curve and to calculate the IC_{50} for each product. The same procedure was performed with fluconazole. Cell viability curve of microorganisms in contact with different concentrations of natural products showed a similar behavior in the screening, which allowed us a random choice of lines for continuity of the work. Thus, the standard strains and clinical isolates of *C. albicans* were

selected (CA INCQS 40006 e CA LM 77) and *C. tropicalis* (CT INCQS 400042 e CT LM 23). Information is shown in Table 2 and Fig. 2. The IC_{50} of products ranged from 1803.02 to 5623.41 μg/mL and cell viability curve image points the hydroethanolic extract of the species as being the most effective because it could reduce a higher percentage of micro-organisms, when compared to the others.

The MIC of the products in this study was determined and standardized as > 8192 μg/mL and cell viability curve, the concentration at which it was observed a marked reduction in the percentage of viable microorganisms. The results of minimum fungicidal concentration showed that, the concentrations tested, no extract showed fungicidal effect, since it failed to remove, but reduce the population of microorganisms of *Candida*. We found that the antifungal effect seen is fungistatic and that minimum fungicidal concentration is > 8192 μg/mL.

One of the main virulence factors of *Candida* is the cell dimorphism, which depends on the environmental conditions in which micro-organisms are found growing. This condition dimorphic is characterized by the ability to alter the cellular morphology, alternating, in a reversible manner, between the shape of yeast and hyphae or pseudohyphae, by issuing a structural extension, a filament, passing a state considered dispersive to invasive, respectively, this being a process commonly observed in candidemia systemic, which can be found in both morphological types, the yeast and filamentous at the same time (Lu et al., 2014).

Based on the results of the MIC, the tests were performed to verify that the extracts influenced the morphological transition of yeast. To this end we prepared a poor environment in nutrients, where it functioned as stressor of microorganisms, stimulating their potential dimorphic. In carrying out the
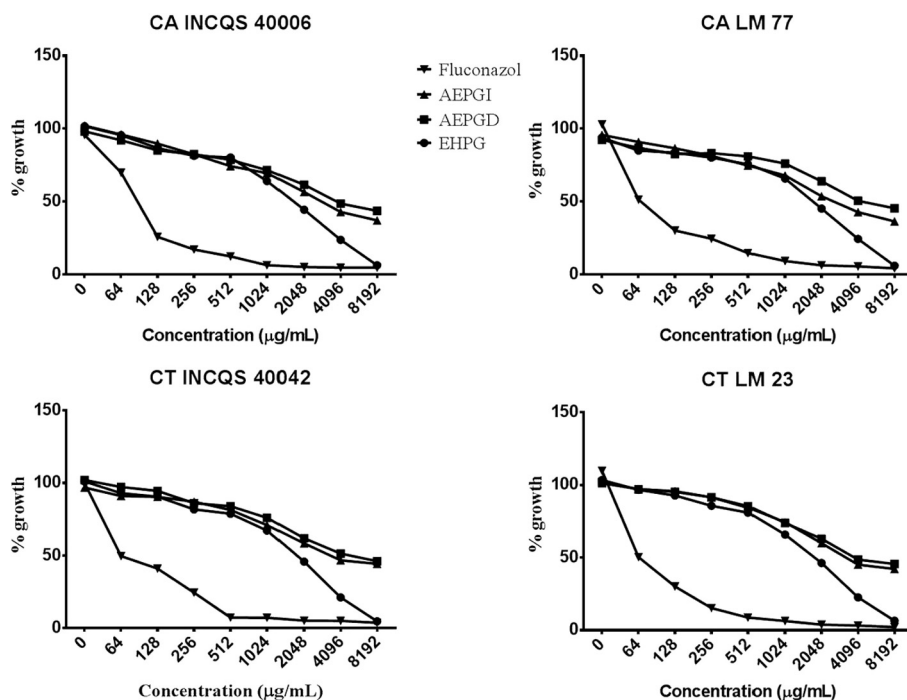

Figure 2 Cell viability curve *Candida* strains under the effect of *Psidium guajava*. CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia; AEPGI: Aqueous Extract of *P. guajava* Infusion; AEPGD: Aqueous Extract of *P. guajava* Decoction; HEPG: Hydroethanolic Extract of *P. guajava*.
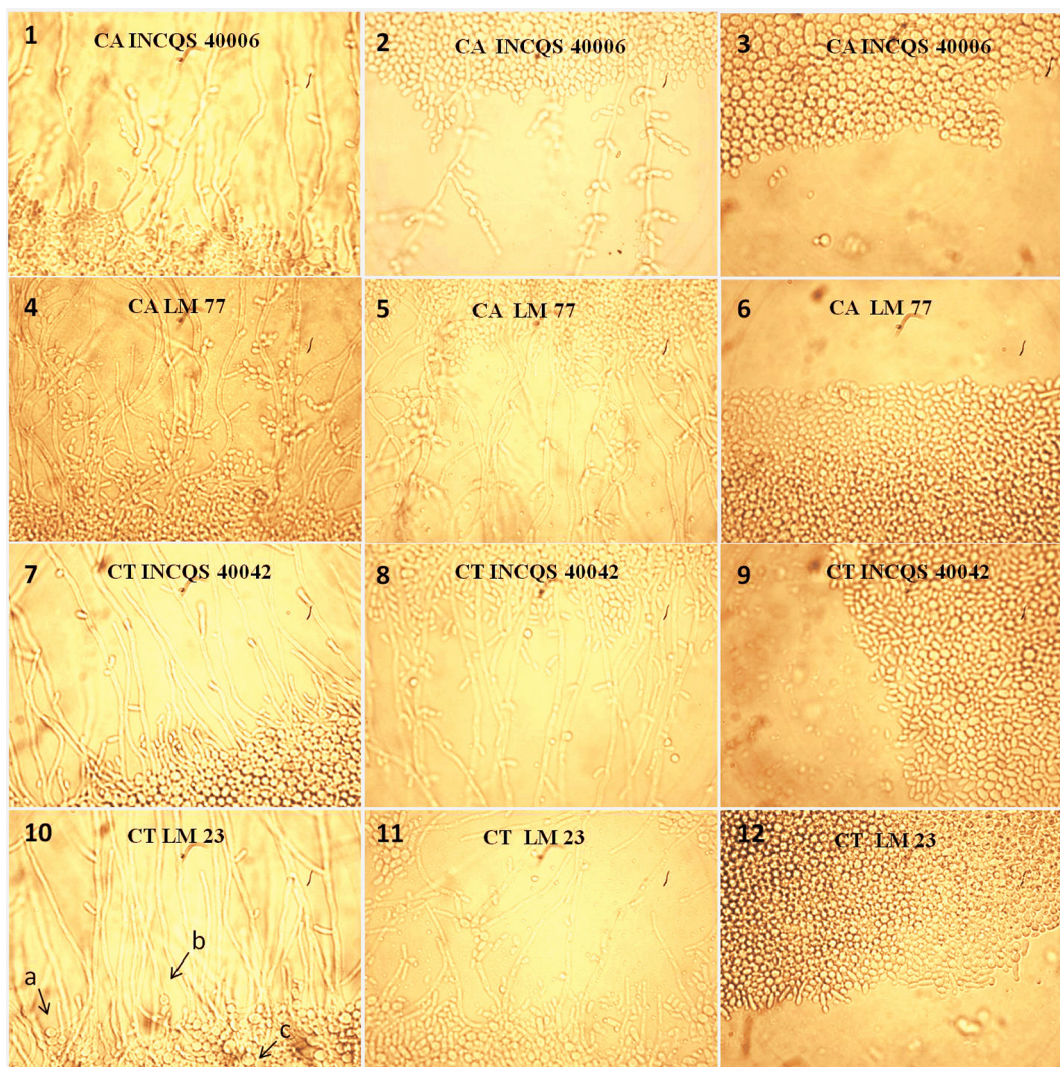
Figure 3 Controls used in micromorphology tests. Cell forms of *Candida*: a: pseudohiphae; b: hiphae; c: yeast; 1, 4, 7 and 10: Growth control; 2, 5, 8 and 11: DMSO 0.05%; 3, 6, 9 and 12: Fluconazole; CA: *Candida albicans;* CT: *Candida tropicalis*; INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia. Images inspected under a light microscope using a 40× objective.

different tests controls were included, one of the growth control, and demonstrated the feasibility of this morphological transition allowed by the nutrient poor environment where the microculture reveals the presence of pseudohyphae, and hyphae. In another control, DMSO was tested at 100% and the maximum concentration is able to prevent the morphological transition (data not shown). However, to show that the change does not exert dimorphic yeast, the DMSO was assayed in concentration contained in dilution of natural products (0.05%), confirmed that the microculture presented microorganisms in accordance with the growth control. The reference drug, fluconazole, was also evaluated and, as the lowest concentration assayed in the test (MIC/2), caused inhibition of emission of filamentous structures. The images of the controls are shown in Fig. 3.

The reading of other microcultivations performed with the extracts in concentrations of 4096, 8192 and 16384 μg/mL showed that they were able to affect the phenotypic plasticity of *C. albicans* and *C. tropicalis* reducing hyphae and

pseudohyphae formation process in so far as their concentrations were increased. At higher concentration, the yeast form prevailed so that, or not verified the presence of filaments, or these filaments were significantly reduced, as can be seen in Figs. 4 and 5.

The guava has been used in Brazil for the treatment of oral diseases, where both the leaves as bark are used in the preparation of tea (for infusion or decoction) to be swallowed or swished still warm, with pretensions to combat thrush and mouth sores, which may be caused by *Candida* strains (Borba and Macedo, 2006; Oliveira et al., 2010). In addition, there is no record that various parts of the plant are used not only to treat thrush but also for the treatment of leukorrhea (Fenner et al., 2006), one of the symptoms of vaginal candidiasis. In South Africa the tea from the leaves and roots of guava by infusion is prepared for the treatment of non-specific venereal diseases (Van Vuuren and Naidoo, 2010) and in Cuba, the use of *P. guajava* in folk medicine was also registered against fungi, where parts of the plant
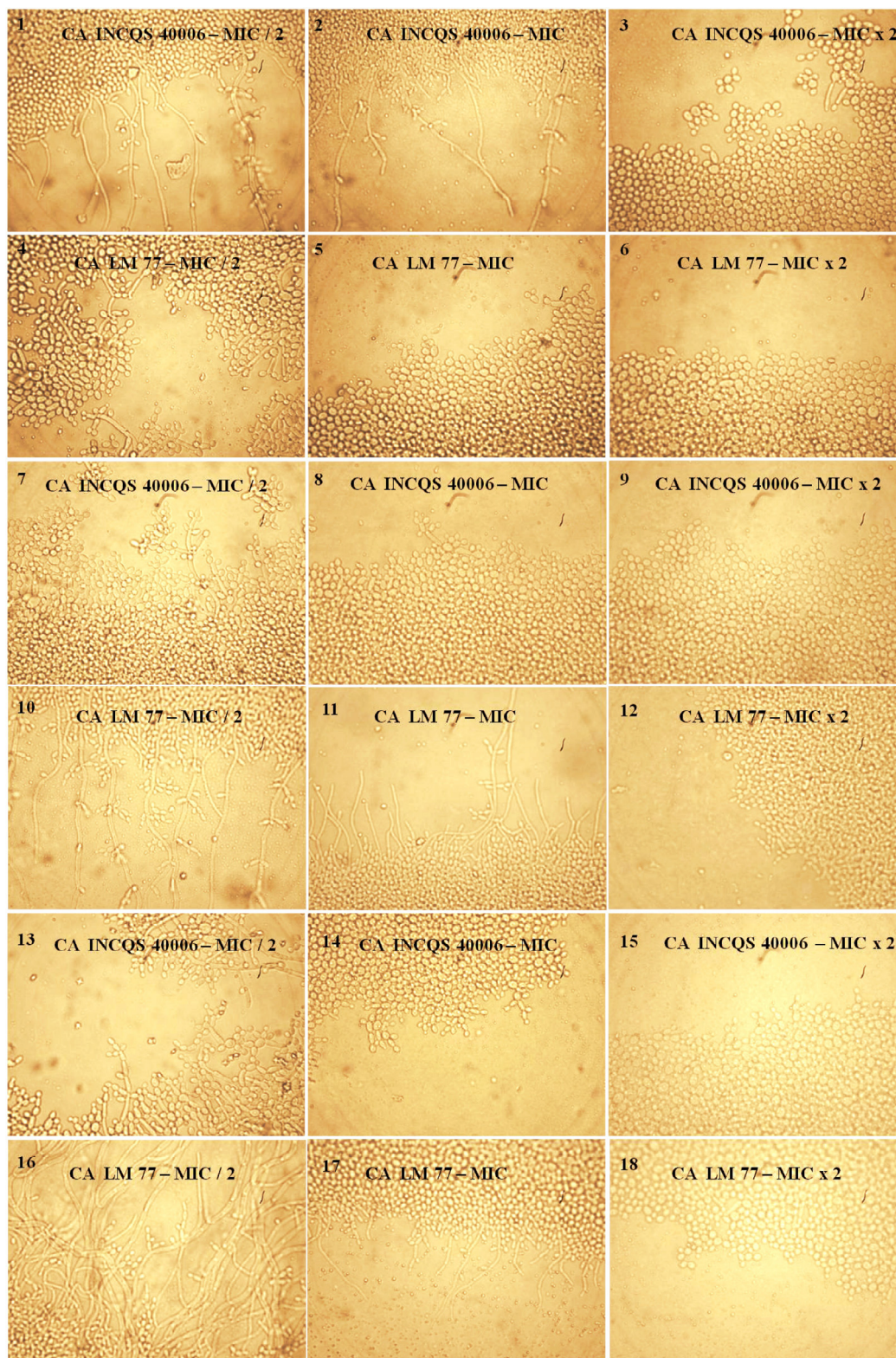
Figure 4 Effect of *Psidium guajava* extracts on the morphology of *Candida albicans*. Concentrations MIC/2, MIC and MIC × 2 (4096, 8192 and 16384 µg/mL, respectively). Aqueous Extract of *P. guajava* Decoction (AEPGD): 1–6; Aqueous Extract of *P. guajava* Infusion (AEPGI): 7–12; Hydroethanolic Extract of *P. guajava* (EHPG): 13–18. INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia. Images inspected under a light microscope using a 40× objective.

Figure 5 Effect of *Psidium guajava* extracts on the morphology of *Candida tropicalis*. Concentrations MIC/2, MIC and MIC × 2 (4096, 8192 and 16384 μg/mL, respectively). Aqueous Extract of *P. guajava* Decoction (AEPGD): 1–6; Aqueous Extract of *P. guajava* Infusion (AEPGI): 7–12; Hydroethanolic Extract of *P. guajava* (EHPG): 13–18. INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia. Images inspected under a light microscope using a 40× objective.

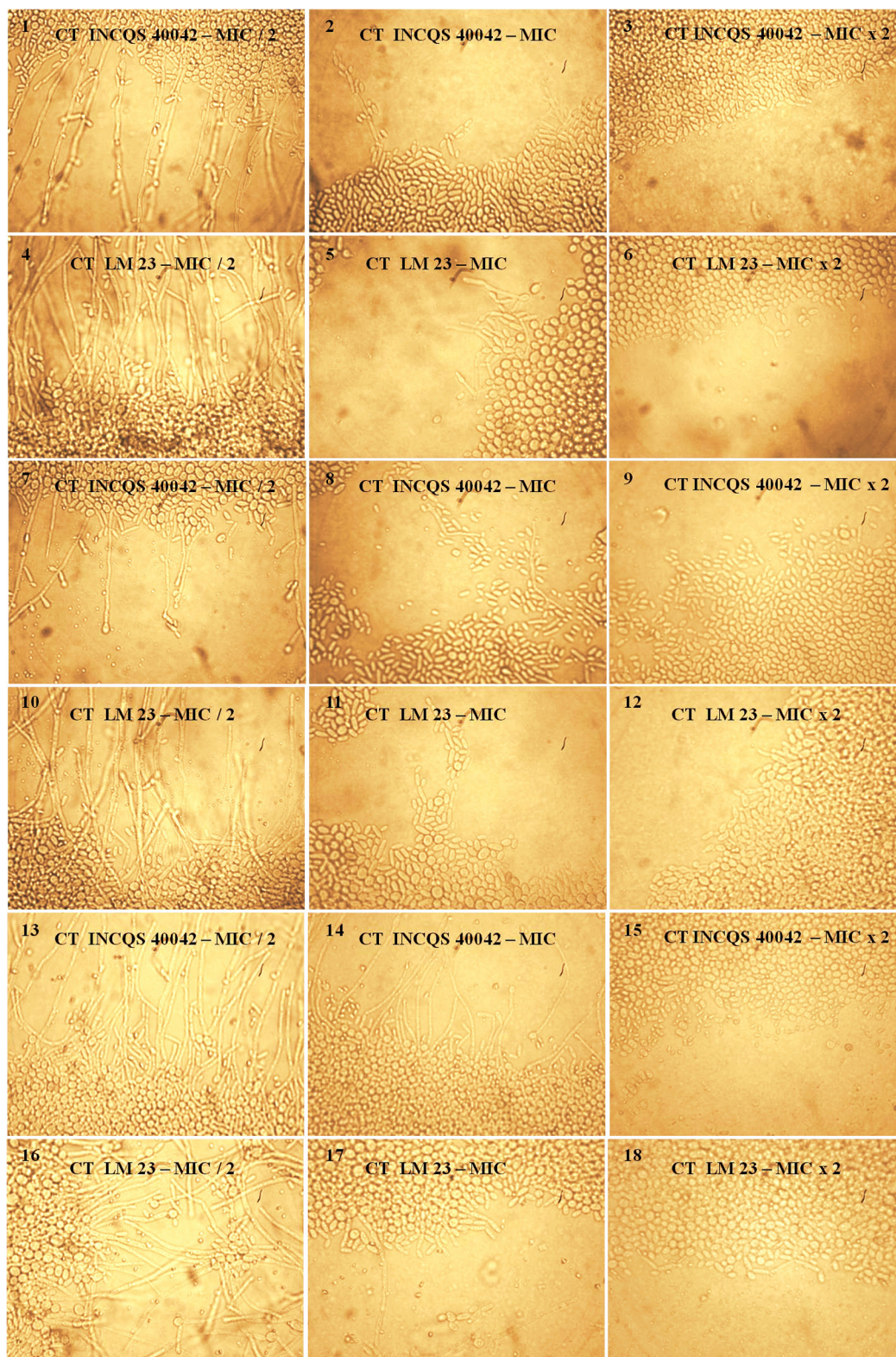are used in the preparation of dye, powder and elixir (Ramírez et al., 2007). Based on these ethnobotanical reports, we can assume that the main form of therapeutic use *P. guajava* against fungi is the topical use, as the natural product is placed directly on the skin or mucosa, used in mouthwash and gargle, in sitz baths and even in tea administration, which when taken favors the contact of the natural product with the intestinal lumen, where the infection causing microorganisms can be accommodated.

If we consider the context of this research, a cup of tea (made by decoction and infusion) with 150 mL of water and 10 g of fresh leaves, will be contained in this volume, just over 4 times the concentration considered as MIC, was able to reduce the percentage of viable microorganisms by direct contact. The same situation is extended to hydroethanolic extract. In 150 mL of tincture is contained 25 times the minimum inhibitory concentration. If relate to the preparation of virulence potential inhibitor in 150 mL of aqueous extracts of plants have about 2 times the concentration at which the filamentous structures of *Candida* have been reduced. In this same volume hydroethanolic extract is contained 12 times the inhibitory concentration dimorphism. Thus a direct contact of tea or tincture prepared in the above relation not only reduces the percentage of viable microorganisms, but also disturbs the process of morphological yeast transition that remains in place after the addition of home-made preparations, neutralizing one of its virulence factors the ability to invade substrates.

Due to the ethnomedicinal use of *P. guajava* observed both in the traditional medicine as complementary and alternative medicine, the plant is now part of the list of medicinal plants of the World Health Organization (WHO). Based on fundamental criteria such as common use in at least two regions of WHO and satisfactory amount of scientific data, this organization has promoted the development of monographs in which relevant information about this and other species of medicinal relevance, was made available to the public access (WHO, 2009). *P. guajava* also reported in national lists of medicinal plants in some countries and is covered in public policy programs focused on primary health care, as occurs, for example, in Brazil (BRASIL, 2009; RENISUS, 2009).

The belief system of some people, low economic power of users, the medications available at minimal cost, the lack of access to another type of therapeutic resource in conflict areas (especially in poor countries), the fact that they are natural products and considered by some to be more effective than allopathic medicines and cause side effects or milder side effects compared to commercial drugs are some of the reasons given to justify the significant use of medicinal plants (Adnan et al., 2014; Khan et al., 2014; OMS, 2002), including the species under study.

Regarding the popular therapy with *P. guajava*, several factors can influence the final result of a treatment as a contact time of natural product with the infection microorganisms, duration of treatment, methods of use, among others.

Although we are talking about parts of a plant that has its fruit habitually used in nutrition for human populations from different locations, the use of tea fresh leaves for infusion has had its cytotoxic potential investigated. The aqueous extract intragastric administration in rats of both sexes (doses of 0.2, 2.0 and 20.0 g/day) for prolonged period (six months) resulted in signs of hepatotoxicity and renal problems as hydronephrosis in males and pyelonephritis, and nephrocalcinosis in females. The LD_{50} of the extract was more than 20.0 g/kg (Attawish et al., 1995).

Almeida et al. (2006) evaluated in vitro the cytotoxicity of tea made by infusing peritoneal macrophages of mice. The infusion of the leaves was prepared and tested both immediately as a few hours after preparation. Soon after preparation, the infusion was added to the culture environment which exhibited 10% mortality rate, increasing to 31.82% after storage at 4 °C for a period of 48 h. After this period, the index rose to 76.18% revealing that the infusion, therefore, presents an immunotoxic effect. In this sense, taking tea in the same day it is prepared can prevent damage being caused to the cells of the immune system, which, according to the authors, may be due to flavonoids oxidation and subsequent release of their derivatives capable to generate radicals free, which would cause toxicity.

People affected with candidiasis usually present with some impairment of their immune responses and in this sense, would be at serious risk, and may further compromise the body's defense case, for lack of such risks, adopt an inadequate alternative therapy. These studies therefore point to a cautious use of tea as much as the duration of treatment, preparation and storage even as the administration of excessive amounts.

The antifungal effect of the species *P. guajava* reported here may be due to the presence of phenolic compounds in the extracts, since they are able to promote both inhibition of growth of *Candida* lineages (Alves et al., 2014; Barros et al., 2013; Candiracci et al., 2011; Tempesti et al., 2012; Vashisth et al., 2013; Candiracci et al., 2012), as well as their filamentous structures resulting from the transition process (Candiracci et al., 2012; Canonico et al., 2014). The percentage of phenolic compounds of the hydroethanolic extract was more pronounced compared to aqueous extracts as well as their potential inhibitor, as can be seen in cell viability curve. However, further investigations are needed to elucidate the mechanisms by which act the extracts and which in fact, are the phytochemicals contained therein, responsible for the observed effect.

P. guajava, in subsequent studies, had its antifungal potential investigated obtaining results favorable for different methodologies (Assunccedil et al., 2013; Jebashree et al., 2011; Mailoa et al., 2014; Suwanmanee et al., 2014), but this was the first report which was investigated and verified its influence on a virulence factor of *Candida*.

4. Conclusion

The use of teas, pastes, plasters and sitz baths prepared from leaves of *P. guajava* (red guava) for different populations had, in this study, their potential bioactive scientifically justified through tests with leaf extracts by direct contact, since, besides provoking a decrease in the population of microorganisms of the genus Candida, affected an important fungal virulence factors, morphological transition, and consequently their invasive potential of tissues. The observed antifungal effect is fungistatic and not fungicidal, since he did not kill the fungi. However, it is important to remember that the existing cultural complex systems in these populations allow different forms of therapeutic preparations with amounts of ingredients that may be different from that used in our tests, it is known that there is no standardization when it comes to the use of medicinal plants. Further studies are needed to

understand the genetic and biochemical processes involved in both dynamic fungistatic as in inhibiting emissions of cell extensions of *C. albicans* and *C. tropicalis* in its virulence.

Declaration of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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