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Research Paper

Antifungal activity of the ethanolic extracts of *Punica granatum* L. and evaluation of the morphological and structural modifications of its compounds upon the cells of *Candida* spp.

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Abstract

Ethanolic crude extracts prepared from the arils and seeds, pericarp, peels and from the whole fruit of *Punica granatum*, known as pomegranate, had their antifungal activity tested against *Candida* spp. The ethanolic crude extracts were analyzed by Mass Spectrometry and yielded many compounds such as punicalagin and galladydilacton. The extracts from the pericarp and peel showed activity against *Candida* spp., with MICs of 125 μg/mL. The effect of pericarp and peel extracts upon the morphological and structure of *C. albicans* and *C. krusei* were examined by scanning and transmission electron microscopy, with the visualization of an irregular membrane and hyphae, formation of vacuoles and thickening of the cell wall. The data obtained revealed potential antimicrobial activity against yeasts cells of the *Candida* genus, and the bioactive compounds could be responsible for changes in cell morphology and structure. The data obtained open new perspectives for future research in continuation to this study, where information such as determination of the site of action of the compounds could contribute to an alternative therapy against these organisms.

Key words: *Punica granatum*, punicalagin, antifungal activity, *Candida* spp., electron microscopy.

Introduction

For centuries, the peels, leaves, flowers and fruits of *Punica granatum* L. (*Punicaceae*), known as pomegranate, have been used to treat many diseases (Gracious Ross *et al.*, 2001). Some studies with extracts of *P. granatum* and compounds have shown high antioxidant (Aviram *et al.*, 2004, 2008; Gil *et al.*, 2000; Reddy *et al.*, 2007; Scalbert, 1991), anti-inflammatory (Kitchen *et al.*, 2004), anticarcinogenic (Adhami and Mukhtar, 2006; Bell and Hawthorne, 2008; Kim *et al.*, 2002; Malik *et al.*, 2005; Malik and Mukhtar, 2006) and antimicrobial activity (Duraipandiyn *et al.*, 2006; Höfling *et al.*, 2010; Pereira *et al.*, 2006; Vasconcelos *et al.*, 2003; Voravuthikunchai *et al.*, 2004).

Among the many compounds present in the *P. granatum* fruit, there are alkaloids, polyphenolics, ellagic acid and gallic acid. The peel of the fruit contains alkaloids and, approximately 20% of tannins, including punicalagin, granatins A and B, gallagyldilacton, casuarinin, pedunculagin, tellimagrandin I and corilagin, which have antibiotic action (Fetrow and Avila, 2000). Compounds such as granatins A and B, punicalagin and punicalin were isolated from the pericarp, and are the main compounds responsible for the antimicrobial activity (Catão *et al.*, 2006). Chemical analyses carried out by Dudonné *et al.* (2009) showed that phenolic compounds of pomegranate presented high levels of hydrolysable tannins, such as punicalin, punicalagin, pedunculagin and punigluconin.

Furthermore, several studies, such as those of Al-Zoreky (2009), Anibal (2010), Duman *et al.* (2009), Duraipandiyan *et al.* (2006), Navarro *et al.* (1996), Reddy *et al.*

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(2007), Vasconcelos *et al.* (2003, 2006), have demonstrated that pomegranate extract inhibits the yeast growth of *Candida* genus.

The yeast cells of *Candida* genus have been studied in order to evaluate the activity of different substances against these microorganisms. *Candida* is a common commensal of the human mucous surface and is an opportunistic pathogen which may undergo conversion from the benign form to the pathogenic form, with many virulence factors that may contribute to the invasion and pathogenicity of these fungi in people with compromised immune systems (Calderone and Fonzi, 2001). Species of *Candida* are prevalent and important pathogens detected in hospitalized patients with nosocomial infections (Pfaller and Diekema, 2007) and mortality remains high despite the advances reached in antifungal therapy (Gudlaugsson *et al.*, 2003). Furthermore, cross resistance may occur when using fluconazole and voriconazole among clinical isolates (Pfaller *et al.*, 2004; Phongpaichit *et al.*, 2005). The aim of this study was to evaluate the antifungal activity of *Punica granatum* extracts and identify the substances involved in this activity by mass spectrometry and observe the structural and morphological effects produced by these extracts by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

Material and Methods

Plant material

Samples of *Punica granatum* L. were collected at the experimental field of CPQBA-Unicamp (Paulinia, Brazil). The corresponding voucher specimen (1223) remains at CPQBA-Herbarium. Dr. Glyn Mara Figueira was responsible for the identification.

Preparation of extracts

Fresh fruit, separated into arils and seeds, pericarp, peel and the whole fruit were submitted to ethanolic extraction (70% ethanol) at room temperature by maceration. The extracts were filtered, the solvent eliminated under vacuum and lyophilized, providing the crude extracts.

Crude extracts were monitored by Thin Layer Chromatography. Spots and bands were visualized by UV irradiation (254 nm and 356 nm) and anisaldehyde and NP/PEG (2-aminoethyl diphenylborinate polyethylene glycol) spray reagentes followed by incubation at 100 °C for 5 min.

Mass spectrometry

Mass spectrometry and Mass/Mass with ionization by electrospray (ESI-MS and ESI-MS/MS) analyzes were performed using the extracts of arils and seeds, pericarp, peel and fruit of the pomegranate, by Q-Tof (Micromass - UK) at Thomson Laboratory (IQ-UNICAMP), under the following conditions: time for each spectra: 1 min; capillary voltage: 3.0 kV; ion source temperature: 100 °C; desolvation temperature: 100 °C; cone voltage: 70 V. Diluted samples were injected through an automatic injection pump (Harvard Apparatus), continuous flow: 10 μL/ min; full spectra: 10 to 2000 *m/z*, with electron ionization of 25 to 70 eV; acquisition mode: scan (m/z 50). Spectra were treated with MassLynx 4.1 software. Samples were protonated $[M+H]^+$, forming adduct ions of sodium $[M+Na]^+$ or potassium $[M+K]^+$.

Microorganisms

The test organisms used proceeding from the Microbiology and Immunology Laboratory, at the School of Dentistry of Piracicaba (FOP/UNICAMP), *Candida albicans* (CBS 562), *C. dubliniensis* (CBS 7987), *C. tropicalis* (CBS 94), *C. krusei* (CBS 573), *C. guilliermondii* (CBS 566), *C. utilis* (CBS 5609), *C. parapsilosis* (CBS 604), *C. lusitaniae* (IZ 06), *C. glabrata* (IZ 07) and *C. rugosa* (IZ 12) (CBS – Centraalbureau voor Schimmelcultures, Dutch collection; IZ: Instituto Zimotécnico, Bank of Superior School of Agriculture "Luiz de Queiroz", ESALQ/USP, Piracicaba, São Paulo, Brazil).

Microbial susceptibility testing

The minimal inhibitory concentration (MIC) of the crude extract for the strains were determined according to the M27-A2 broth microdilution reference procedure of CLSI (2002), with modifications, at a final inoculum of 5.0 x 10^3 CFU/mL, using BHI (brain heart infusion) broth. Serial dilutions of the crude extract, ranging from 7.8 to 1000 μg/mL, were carried out in a microdilution plate (96 wells) containing 100 μL of BHI. The inoculums were then added to each well. The microplates were incubated at 37 °C for 48 h. MIC was defined as the lowest concentration which resulted in inhibition of visual growth. Minimal fungicidal concentrations were determined by subculturing 10 μL of the culture from each negative well and from the positive control on Sabouraud Dextrose Agar (SDA). All these analyses were performed in triplicate. Nistatin was used as control, ranging from 1.9 to 250 μg/mL.

Scanning electron microscopy

Inoculum exposed to concentration ranging from 1000 μg/mL to 125 μg/mL of crude extract at 37 °C for 24 h and control cells were collected, washed twice with PBS solution, and centrifuged for 5 min at 3000 rpm. The pellet was fixed in 2.0% glutaraldehyde (Sigma-Aldrich, USA) in phosphate buffer, pH 7.4. Dehydration was achieved by 10 min sequential ethanol washes. A drop was placed onto lab-tek slides (BD) and coated with gold in a BAL-TEC SCD 050 (Balzers Liechtensteis) apparatus and assessed by a JSM-5600 Lv JEOL (Tokyo, Japan), at 15 kV.

Transmission electron microscopy

Cells exposed to concentration ranging from 1000 μg/mL to 125 μg/mL of crude extract at 37 °C for 24 h

and control cells were collected, washed twice with PBS solution, and centrifuged for 5 min at 3000 rpm. The pellet was fixed with 2.0% glutaraldehyde (Sigma-Aldrich, USA) in a 0.1 M cacodylate buffer, pH 7.2. At the electron microscopy laboratory of FOP/UNICAMP, under the supervision of Prof. Dr. Pedro Duarte Novaes, postfixation was carried out in 2% OsO_4 M cacodylate buffer (pH 7.2) for 1 h at room temperature. Thereafter, the cells were dehydrated in acetone and embedded in SPURR. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in EM-10 (Zeiss) transmission electron microscopy at NAP/MEPA (Support Research Nucleus / Electron Microscopy Applied to Agriculture) ESALQ/USP (Superior School of Agriculture "Luiz de Queiroz"/ University of São Paulo), under the supervision of Prof. Dr. Elliot Watanabe Kitajima.

Results

Antifungal activity of arils and seeds, pericarp, peel and fruit extracts

The results of *P. granatum* extracts, obtained from the whole fruit, from arils and seeds, pericarp and peel, are demonstrated in Tables 1 and 2. As shown in Table 1, after 24 h of growth it was possible to observe the activity of pericarp, peel and fruit extracts and the resistance of all strains to the aril and seed extract. Pericarp extract presented a MIC of 31.5 μg/mL to *C. parapsilosis*, 62.5 μg/mL to *C. utilis, C. lusitaniae* and *C. glabrata*, and 125 or 250 μg/mL for the others *Candida* species, showing a greater activity than the other extracts.

After 48 h of growth, the extracts presenting greater inhibitory activity were those obtained from pericarp and from the peel with MIC of 125 μg/mL (*C. krusei, C. guilliermondii, C. parapsilosis, C. lusitaniae* and *C. rugosa*) and 250 μg/mL (*C. albicans, C. dubliniensis, C. tropicalis* and *C. glabrata*) (Table 2).

After the end of 48 h, samples and controls were inoculated on Sabouraud Dextrose Agar (Difco) and the growth of samples and controls was observed, showing that these extracts had a fungistatic effect whereas nistatin had a fungicidal effect.

Mass spectrometry analyses of samples

Monitoring the extracts using Thin Layer Chromatography and NP/PEG reagent showed the presence of phenolic compounds (data not show). The results obtained from the Mass Spectrometry analyses of arils and seeds (data not shown), fruit (data not shown), pericarp and peel extracts of *Punica granatum* revealed compounds that were identified by their molecular weight (MW) and structure, using data from literature (Lansky and Newman, 2007), in addition to other compounds that were not identified, due to variations between the mass-to-charge of the compounds.

Table 1 - Antifungal activity (MIC - μg/mL) of ethanolic extracts of *P. granatum* (24 h).

Microorganisms	Arils + Seeds	Pericarp	Peel	Fruit	Nistatin
C. albicans	*	125	125	500	3.9
C. dubliniensis	*	125	250	250	3.9
C. tropicalis	*	250	250	500	1.9
C. krusei	*	125	125	250	3.9
C. guilliermondii	*	125	125	250	1.9
C. utilis	*	62.5	125	500	1.9
C. parapsilosis	*	31.5	125	125	3.9
C. lusitaniae	*	62.5	62.5	125	1.9
C. glabrata	*	62.5	125	250	3.9
C. rugosa	*	125	62.5	250	3.9

*: MIC values ≥ 1000 μg/mL.

Table 2 - Antifungal activity (MIC - μg/mL) of ethanolic extracts of *P. granatum* (48 h).

Microorganisms	Pericarp	Peel	Fruit	Nistatin
C. albicans	250	250	1.000	3.9
C. dubliniensis	250	250	500	3.9
C. tropicalis	250	250	500	1.9
C. krusei	125	125	250	3.9
C. guilliermondii	125	125	250	1.9
C. utilis	250	500	500	1.9
C. parapsilosis	125	125	250	3.9
C. lusitaniae	125	125	250	1.9
C. glabrata	250	250	500	3.9
C. rugosa	125	125	250	3.9

The data for the pericarp extract using Mass Spectrometry (Figure 1) revealed over 30 compounds, including: melatonin (MW: 221), delfinidin 3-*O*-glucoside (MW: 465), punicacortein A (MW: 635), punicacortein B (MW: 635), pedunculagin (MW: 823) and tellimagrandin (MW: 823). Compounds above 40% were: glucose (MW: 219), citric acid (MW: 219), caffeic acid (MW: 219), punicic acid (*cis*-9, *trans*-11, *cis*-13 octadecatrienoic acid) (MW: 277), ellagic acid (MW: 303), quercetin (MW: 303), delphinidin (MW: 303), gallagyldilacton (MW: 603) and punicalagin (MW: 1123).

Figure 2 shows the analyses of the peel sample with over 30 compounds detected, those identified included: punicacortein A (MW: 635), punicacortein B (MW: 635), corilagin (MW: 635), punicacortein A (MW: 673), punicacortein B (MW: 673), corilagin (MW: 673). Compounds above 40% were: glucose (MW: 219), citric acid (MW: 219), caffeic acid (MW: 219), punicic acid (*cis*-9, *trans*-11, *cis*-13 octadecatrienoic acid) (MW: 277),

pedunculagin (MW: 823), tellimagrandin (MW: 823). Punicalagin (MW: 1123) presented 100% of detection.

Effect on the morphology and structure of *Candida*

The effect of pericarp and peel extracts on the morphology and structure of *Candida* was examined by scanning and transmission electron microscopy. Analyses of scanning electron microscopy showed control cells with a homogeneous and regular surface (Figure 3A, E) and normal hyphae (Figure 3C). Irregular surface aggregated cells with a production of viscous material were seen in yeasts treated with crude extract (Figure 3B, F) and hyphae with desquamated (thin arrow) and rupture in the cell (large arrow) (Figure 3D). Transmission electron microscopy

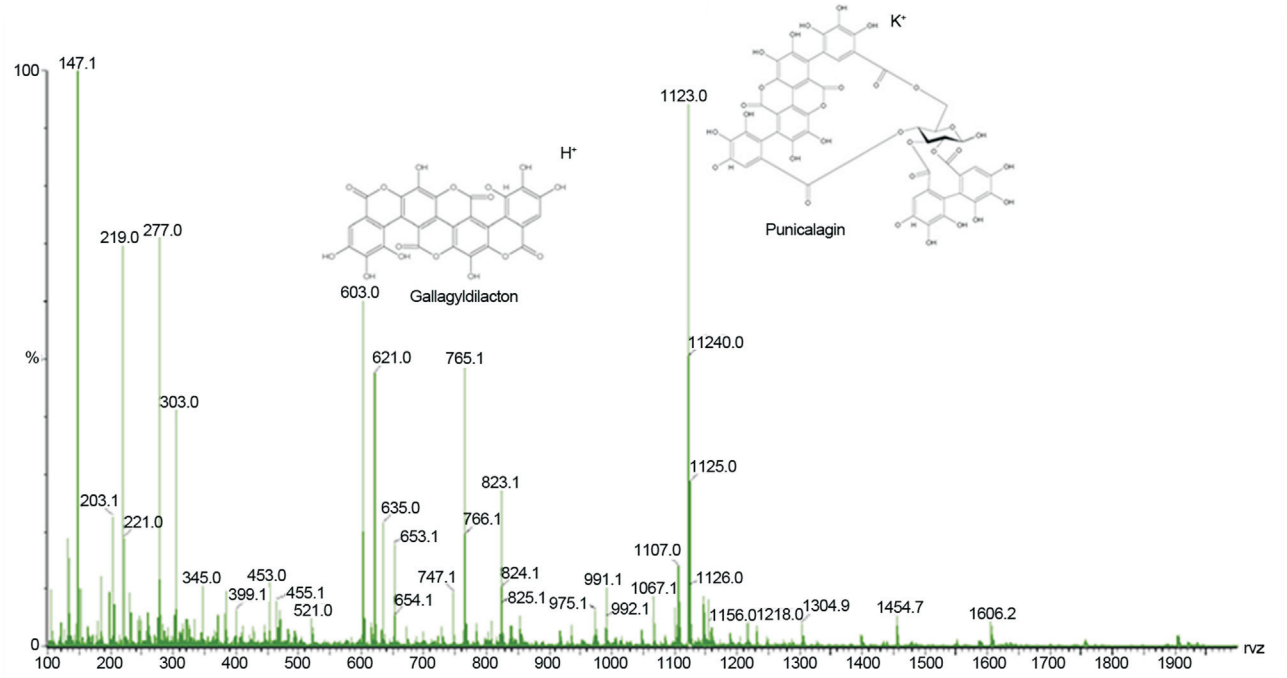

Figure 1 - Monitoring of chemical classes by ESI(+)-MS of the pericarp sample (Q-Tof).

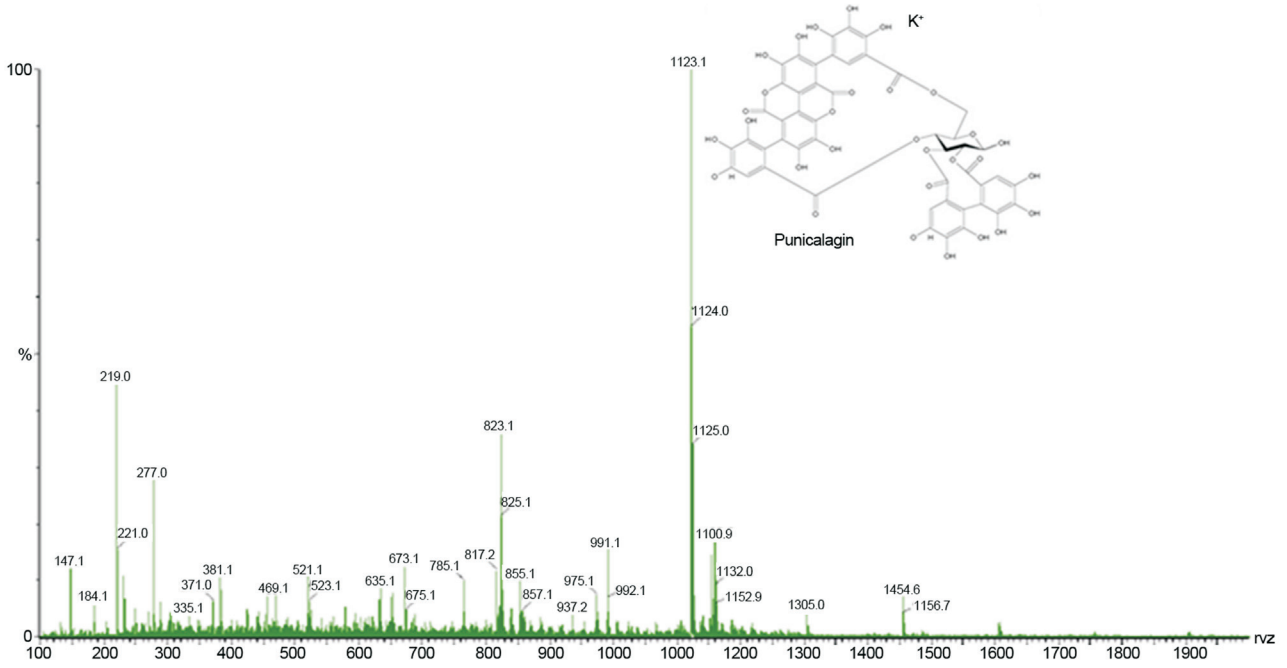

Figure 2 - Monitoring of chemical classes by ESI(+)-MS of peel sample (Q-Tof).

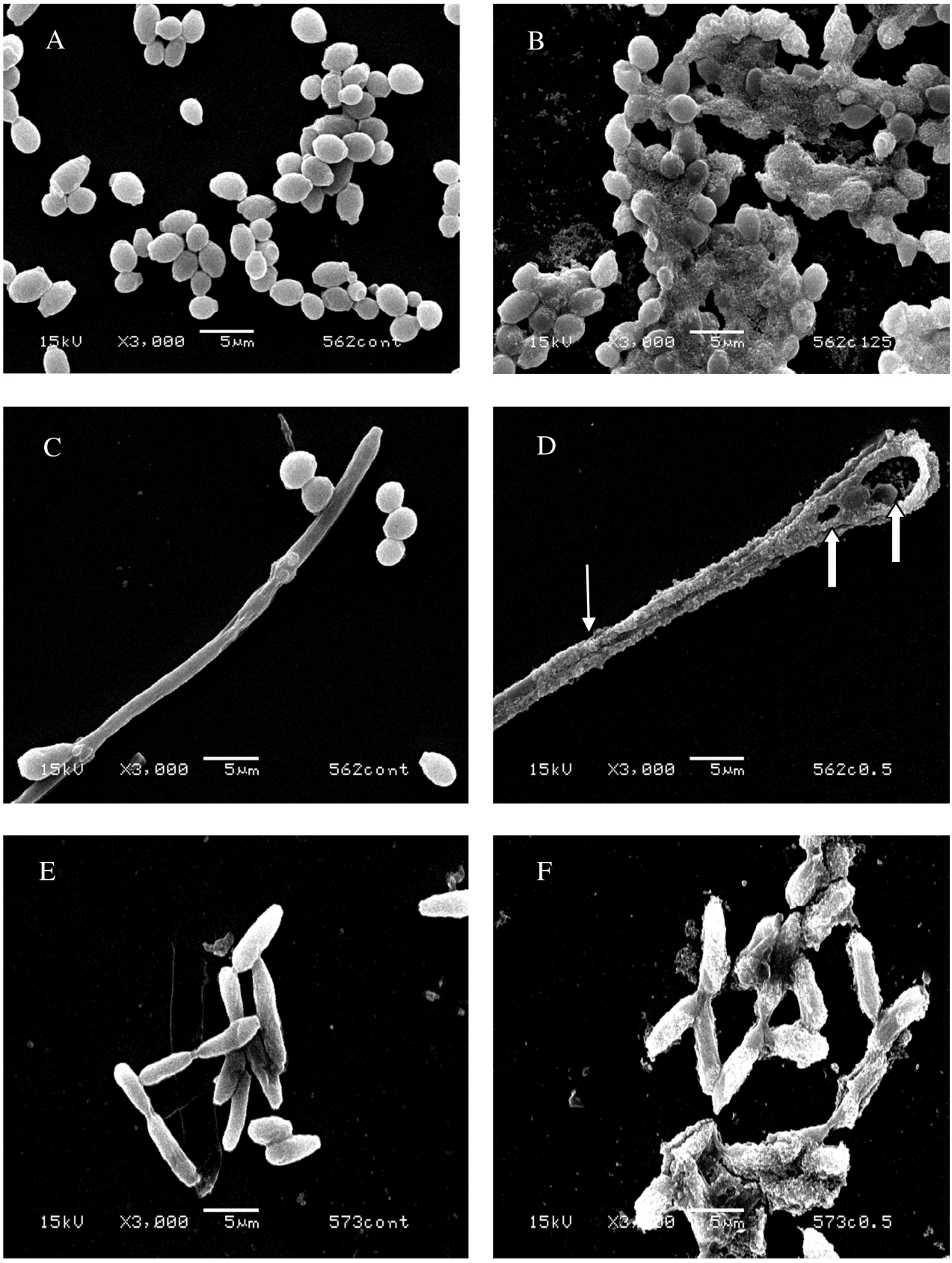

Figure 3 - Scanning electron microscopy: A) *Candida albicans* control cells. B) Cells treated with 125 µg/mL of peel extract. C) *Candida albicans* hyphe: control. D) Cells treated with 500 µg/mL of peel extract with desquamation (thin arrow) and rupture in the cell (large arrows). E) *Candida krusei* control cells. F) Cells treated with 500 µg/mL of peel extract. 3000X.

showed control cells surrounded by a thin and regular cell wall, with a normal cytoplasmic membrane (Figure 4A and Figure 5A and C). Treated cells showed a thickened and irregular cell wall, with more-electron-dense out layer (arrows), deformed cell walls (4B, C and E, large arrow) and changed of cytoplasmic membrane (Figure 4B to F and Figure 5B and D).

Discussion

Due to the increasing development of drug resistance and to the undesirable effects caused by the existing antifungal agents, searches for new antimicrobial compounds has been the focus of a number of studies (Pfaller *et al.*, 2004). The interest for natural products has increased during the years, and medicinal plants have been identified as sources for bioactive compounds. These compounds have been isolated and submitted to detailed analyses to identify their mechanisms of action and target sites (Ishida *et al.*, 2006).

Duman *et al.* (2009) investigated the antimicrobial activity of six varieties of *Punica granatum*, correlating the responses to the phytonutrient properties, such as total phenolic and anthocyanin compounds, and found positive results regarding the inhibition of Gram-positive and Gram-negative microorganisms, and *Candida albicans*, linking these positive results to the antioxidant capacity of this plant. Kakiuchi *et al.* (1986) and Pereira *et al.* (2006) demonstrated the antimicrobial activity of *P. granatum Linn.,* the potential to inhibit the growth of dental bacterial biofilm and polyglucan synthesis, by acting upon the adherence mechanisms of biofilm organisms to the surface of the tooth.

The extracts obtained from pericarp and peel presented potential inhibitory activity, with a MIC of 125 μg/mL. These data indicate that the activity of *P. granatum* probably occurs in the compounds present in the pericarp and peel of this fruit. Analyses of the four extracts of *P. granatum* detected the presence of similar substances, such as melatonin and simple sugars. Compounds with MW of 219, present in 100% of the arils and seeds (data not shown) and in all of the extracts, appeared to exert little or no activity against yeasts once the extracts of arils and seeds showed no activity against the sample tested, and although the extract of the fruit inhibited the samples, the MIC was higher.

The samples of pericarp and peel extract, that presented better results against *Candida* species, had a high concentration of the punicalagin compound as a differential, in addition to other tannins, such as pedunculagin and tellimagrandin. The pericarp sample also revealed the tannin gallagyldilacton, a substance which was not detected in significant quantity in the other samples.

Therefore, punicalagin and gallagyldilacton are apparently the major compounds involved in antifungal activity observed in the extracts of *P. granatum*. The possibility of occurring synergism between the compounds is a fact that cannot be discarded as several substances such as simple sugar and conjugated fatty acids are present in large quantities.

These data corroborate the observations of some searches. Catão *et al.* (2006) and Fetrow and Avila (2000) indicated tannins, such as punicalin, punicalagin, gallagyldilacton, pedunculagin, tellimagrandin I and corilagin, to be responsible for the antimicrobial activity. Endo *et al.* (2010) observed elevated activity of the isolated compound punicalagin against *C. albicans* and *C. parapsilosis*, indicating this substance as a potent antifungal agent, without however, elucidating the mechanism of action. Vasconcelos *et al.* (2003) assigns to the tannins, the ability to inhibit the growth of yeast *Candida* species due to their action in the cell, specifically in the cell membrane, precipitating proteins.

As observed by scanning electron microscopy, the pericarp and peel extracts showed morphological alterations on the yeasts tested, in addition to cell aggregation and growth inhibition. The control sample (Figure 3A) presented a regular and homogeneous appearance, with a normal budding profile at the cell and hyphae, and treated cells presented an irregular cell wall, with viscous material on surface, in addition to rupture of the hyphae with desquamation (3D).

Data obtained from transmission electron microscopy showed interference of the extracts upon the structure of yeast, with alterations of the cell wall, which became thicker, presenting deformed cell, with changed in cytoplasmic membrane, cell wall composed of more-electron-dense layer, seemed to be multilayer cell wall (Figures 4B-F, 5B and D) and an abnormal thickness of the septum was observed between mother and daughter cells, with no clear separation between the cells (Figure 4E), indicating that the extracts caused serious damage to the cell structure of the yeasts. Scalbert (1991) proposed different mechanisms to explain tannin antimicrobial activity. These include (i) inhibition of extracellular microbial enzymes; (ii) deprivation of substrates and metal ions required for microbial growth and (iii) direct action on microbial metabolism through inhibition of oxidative phosphorylation. In addition, Haslam (1996) proposed that tannins are able to complex with other molecules, including macromolecules such as proteins and polysaccharides.

A first step towards the discovery of new drugs is to identify and validate specific molecular targets. With the increasing knowledge of biochemistry and microbial genetics, it is now possible to identify key microbial enzymes, receptors and processes at molecular levels that could rep-

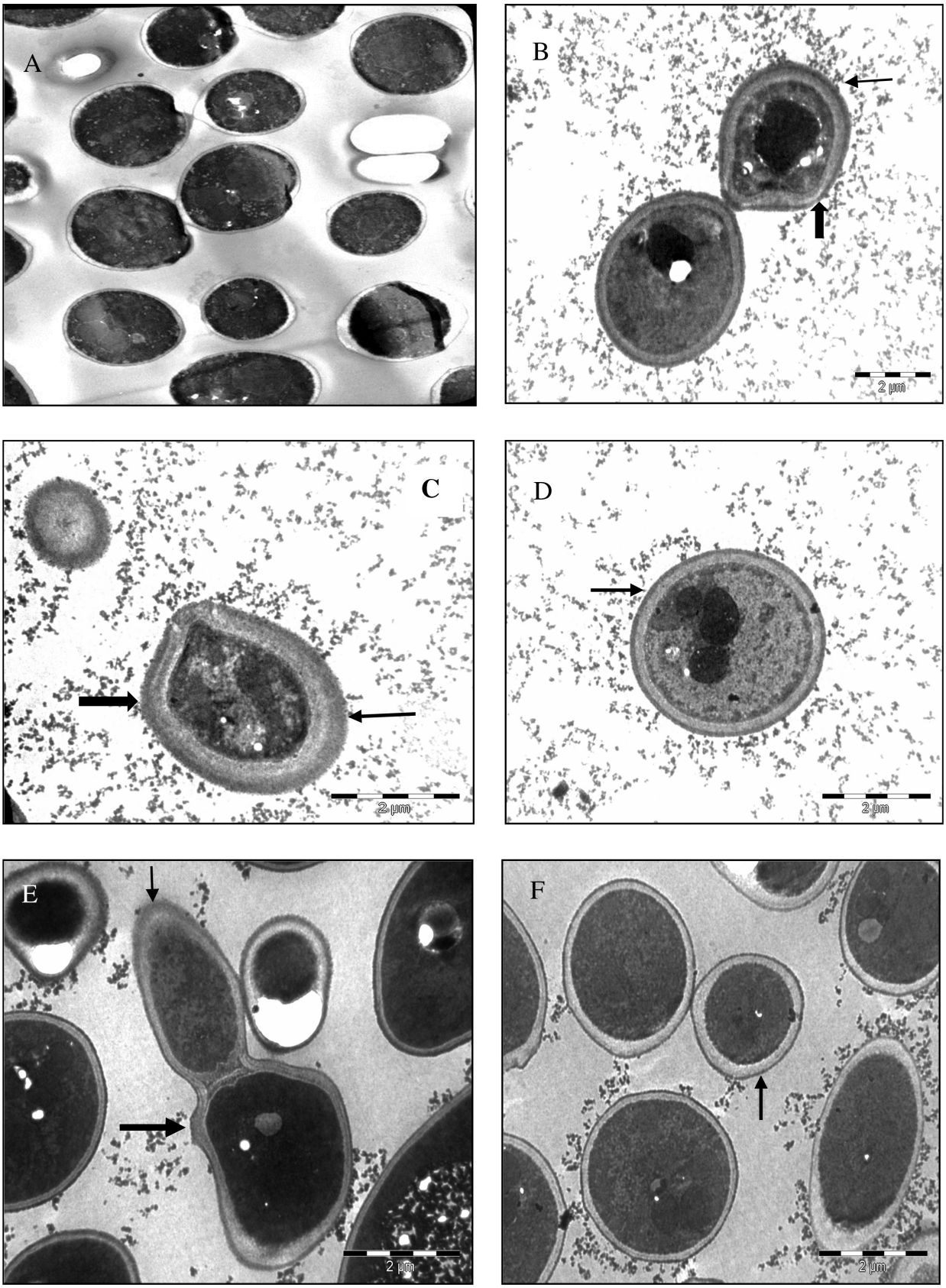

Figure 4 - Transmission electron microscopy: A) *Candida albicans* control cells (7000X). B) Cells treated with 1000 µg/mL of pericarp extract with irregular cell wall (arrow) (3000X). C) Cells treated with 125 µg/mL of pericarp extract with irregular cell wall (arrow) (4400X). D) Cells treated with 125 µg/mL of pericarp extract (4400X). E and F) Cells treated with 500 µg/mL of pericarp extract with irregular cell wall (arrow) (4400X).

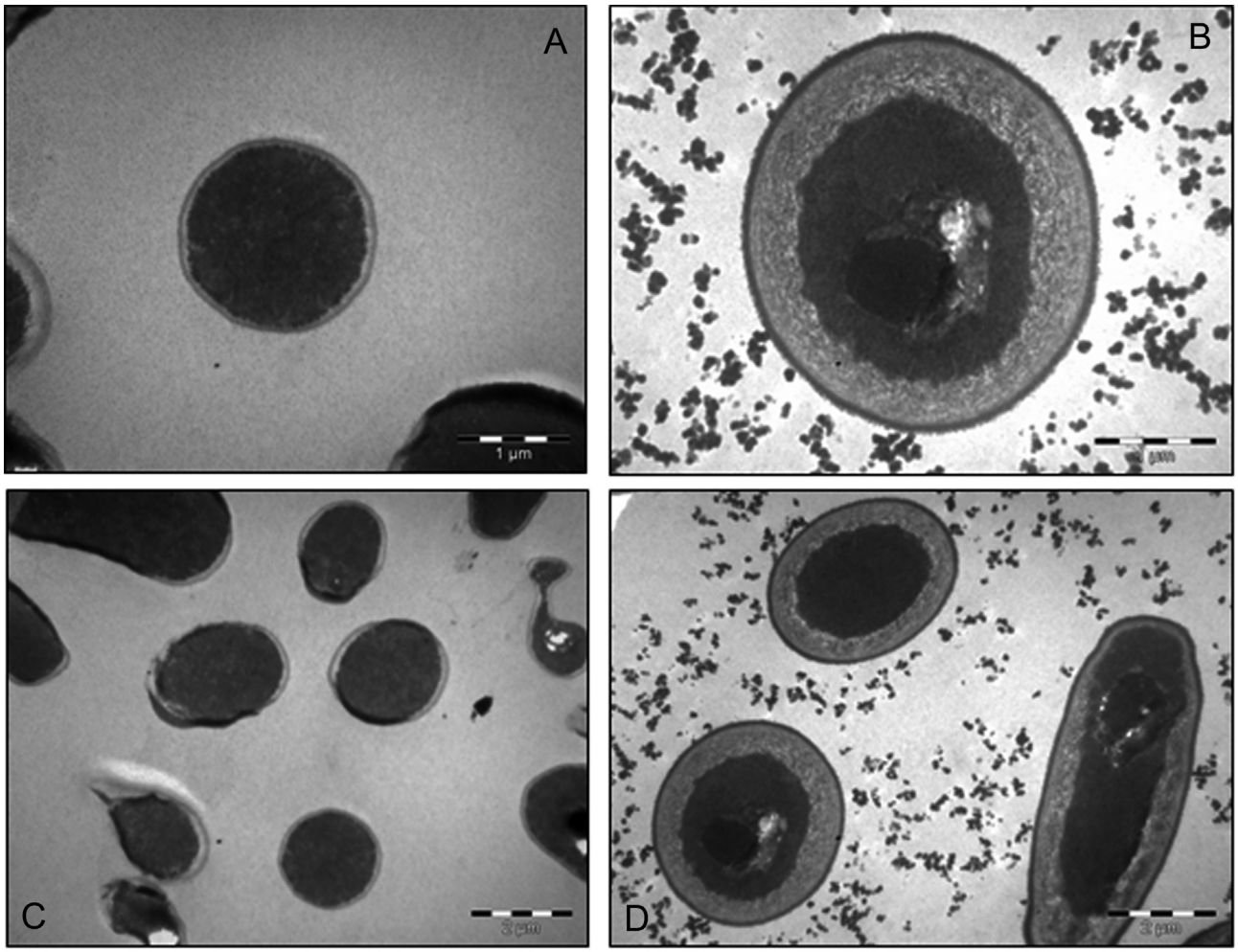

Figure 5 - Transmission electron microscopy: A) *Candida krusei* control cells (7000X). B) Cells treated with 500 µg/mL of peel extract with thickened cell wall (7000X). C) *Candida krusei* control cells (3000X). D) Cells treated with 500 µg/mL of peel extract with thickened cell wall (3000X).

resent targets for the action of drugs. Compounds that block the function of these targets and which may inhibit microbial growth can now be identified. Surveys of microbial genomics and proteomics allow a more rapid advance between the microbial structural information and interaction of the drug, created by computer programs (Kitchen *et al.*, 2004).

Data obtained in our research with substances of *Punica granatum* showed antimicrobial capacity against yeast cells of *Candida* genus, with a MIC of 125 µg/mL. Bioactive compounds (tannins) present in the pericarp and peel showed antifungal activity. Compounds of this family of substances could be involved in the antifungal action detected by mass spectrometry. These bioactive compounds could be responsible for changes in cell morphology, inhibiting growth, producing viscous material and rupturing the cells. These results represent new perspectives for future research in continuation to this study, where information such as the determination of the site of action of the compounds could contribute to the management of alternative therapies against these organisms.

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