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Punica granatum L. Extract Antibiofilm Action against *Acinetobacter baumannii* Carbapenem-Resistant and Biocompatibility over Human Keratinocytes

Ação Antibiofime de Extrato de *Punica granatum L* Contra *Acinetobacter baumannii* Resistente a Carbapnêmicos e Biocompatibilidade em Queratinócitos Humanos

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Abstract

Acinetobacter baumannii is a multi-drug resistant microorganism. The objective of this study was to evaluate the antimicrobial and antibiofilm action of the pomegranate natural extract against eight strains of multidrug resistant *Acinetobacter baumanii* and to assess the extract cytotoxicity in a culture of Human Keratinocytes (HaCat). Broth microdilution method was used to determine the minimum inhibitory and minimum microbicidal concentrations of the extracts. The extract antibiofilm action was analysed by the MTT colorimetric test. The cytotoxicity evaluation was performed by the MTT colorimetric test, which analysed the mitochondrial cellular action, after contact of the extract for 5 min. The results were statistically analysed by ANOVA and Tukey test with a significance level $\alpha \leq 0.05$. *Punica granatum L.* (pomegranate) extract had antimicrobial action on all the 8 clinical strains of *Acinetobacter baumannii* evaluated. The extract showed a significant reduction in metabolic action in biofilm for all the strains, with results statistically different from growth control ($p \leq 0.05\%$). *P. granatum* extract applied for 5 min on human keratinocytes (HaCat) promoted cell viability in all the tested concentrations. The pomegranate extract is effective in reducing the multidrug-resistant clinical strains of *Acinetobacter baumanni* and is biocompatible.

Keywords: Pomegranate. Phytotherapy. HaCaT Cells.

Resumo

Acinetobacter baumannii é um microrganismo multirresistente. O objetivo deste estudo foi avaliar a ação antimicrobiana e antibiofilme do extrato natural de romã contra oito cepas de A. baumanii multirresistente e avaliar a citotoxicidade do extrato em uma cultura de queratinócitos humanos (HaCat). O método de microdiluição em caldo foi utilizado para determinar as concentrações inibitórias mínimas e microbicidas mínimas dos extratos. A ação antibiofilme do extrato foi analisada pelo teste colorimétrico MTT. A avaliação da citotoxicidade foi realizada pelo teste colorimétrico MTT, que analisou a ação celular mitocondrial, após contato do extrato por 5 min. Os resultados foram analisados estatisticamente por ANOVA e teste de Tukey com nível de significância $\alpha \leq 0,05$. O extrato de Punica granatum L. (romã) apresentou ação antimicrobiana em todas as 8 cepas clínicas avaliadas de A. baumannii. O extrato apresentou redução significativa na ação metabólica no biofilme para todas as linhagens, com resultados estatisticamente diferentes do controle de crescimento ($p \leq 0,05\%$). O extrato de P. granatum aplicado por 5 min em queratinócitos humanos (HaCat) promoveu viabilidade celular em todas as concentrações testadas. O extrato de romã é eficaz na redução das cepas clínicas multirresistentes de Acinetobacter baumanni e é biocompatível.

Palavras-chave: Romã (Fruta). Fitoterapia. Células HaCaT.

1 Introduction

Acinetobacter baumannii is a multi-drug resistant microorganism, and it was reported as a critical priority pathogen on the global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics of the world health organization "WHO"[1] and more recently on the Indian priority pathogen list by the WHO country office for India[2]. Its resistance mechanism is related to genome plasticity, efflux pumps, production of beta-lactamases and reduced permeability[3,4] explaining its high incidence rate[5].

It causes bloodstream, respiratory tract and nosocomial infections leading to a high risk of mortality and morbidity in debilitating patients in the intensive care units (ICUs) because of prolonged hospitalization, antibiotics indiscriminate use[4,6] and protective skin loss in burned patients[7]. Furthermore, it is detected in endodontic and periodontal infections[8,9].

Carbapenem-sulbactam combination and other were reported as effective antibiotic combination over *A. baumannii*[10] in addition to other combinations, however, the emergence of new multi-drug strains complicate its disinfection[11,12]. Polymyxin B and polymyxin E (colistin) gained new interest to combat multi-drug resistant strains of *A. baumannii*[13] and even to treat resistant endodontic infections[14,15], however, colistin nephrotoxicity is still discussed[16]. Thus, an increased need for alternative medicines is urgent.

Diverse herbal medicines were indicated to combat a variety of resistant microorganisms[17-19] and more specifically over *A. baumannii*[20]. Pomegranate "Punica granatum L", belongs to family Lythraceae, subfamily Punicoideae, is widely used

as an alternative medicine because of its antioxidant, anti-inflammatory, antibacterial, immune modulatory, antifungal and antitumor action[21,22]. Punica granatum L presented antimicrobial effect over *A. baumannii*[23], *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa*[24,25]. Still, more studies are required to evaluate the effect of Punica granatum L extracts over the new multi-drug resistant *A. baumannii* strains.

The aim of this study was to evaluate the antimicrobial and antibiofilm action of Punica granatum L extracts over eight strains of the multidrug resistant *A. baumanii,* as well to evaluate its cytotoxicity over culture of human keratinocytes (HaCat). The null hypothesis was that the extracts are not effective over the multidrug resistant *A. baumanii* strains, and it is cytotoxic over culture of human keratinocytes.

2 Material and Methods

2.1 Bacterial Strains and Plant extract

Eight carbapenem-resistant strains of *A. baumannii* were provided by the ValeClin Clinical Analysis Laboratory (São José dos Campos, SP, Brazil). The clinical strains identification and the antibiogram were performed by the semi-automated MicroSCAN 4 system. The resistance profile of the selected strains showed resistance to carbapenems, meropenem and imipenem. *Punica granatum* glycolic extracts (Mapric, São Paulo, SP, Brazil) were obtained of the pomegranate fruit including rind and seeds at a concentration of 200 mg / mL in propylene glycol.

2.2 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Microbicide Concentration (CMM)

To determine MIC value, broth microdilution method based on the Clinical and Laboratory Standards Institute (CLSI), standard M7-A10 (2015) was used[26]. A standardized bacterial inoculum of each strain was prepared of culture seeded on BHI agar (Himedia, Mumbai, India) after 24 h of incubation at 37 °C as detailed previously[20] and adjusted at 625 nm in a concentration of 10^6 cells/mL by the spectrophotometer (V-5000, Shanghai Metash Instruments Co., Ltd, China). The test was performed in 96-well microplates (TPP, Trasadingen, Switzerland), performing the serial dilution of pomegranate extract in Mueller Hinton broth (Himedia, Mumbai, India), testing 10 different concentrations, ranging from 50 mg/mL to 0.097 mg/ml (n=8 for each group). All the experimental groups were tested in duplicate for each strain.

The plates were incubated for 24h at 37 °C after inoculation of 100 µL of the bacterial suspension. Then, the MIC value was determined where the well with the lowest concentration of the extract that did not show turbidity. For the CMM determination, 10 µL of the extract at MIC value was seeded onto Brain Heart Infusion Brot (BHI) agar, as well as 10 µL of a concentration above and below MIC value. After 48h of incubation, CMM was determined at the lowest sown concentration that did not show colony growth.

2.3 Evaluation of the extract antimicrobial action on monotypic biofilms

For the biofilm assembly, colonies of the respective strains were diluted onto Brain Hearth Infusion Broth (Himedia, Mumbai, India), preparing standardized solutions in the concentrations of 10^7 CFU / mL (625 nm wavelength), obtaining the optical density of 0.080 (+ - 0.010) (Micronal B- 582, São Paulo, Brazil). Then, 200 µL of microbial suspensions standardized were added in 96-well microplate (TPP, Switzerland) and incubated for 48 hours, with the culture medium replacement in 24 hours.

2.4 Treatment and Metabolic action of microorganisms by the MTT colorimetric test

After biofilms formation, each strain was treated separately with 200 µl of each extract for 5 min, in the three highest concentrations verified as MIC (50, 25 and 12.5 mg/mL). All the plates were incubated at 37 °C. BHI broth was used as a negative control group. Then, biofilms were washed with sterile 0.9% saline solution, to remove the bacterial cells affected by the treatment, then they were sent to MTT test using 100 µL of the MTT solution (3-(4,5-Dimethylthiazol-2-yl) -2,5-DiphenyltetrazoliumBromide) (Sigma-Aldrich,Missouri – USA)[20]. Two independent experiments were carried out, with 6 repetitions each, totalling n = 12 for each experimental group. The optical density "OD" of each well was measured by the microplate reader at 570 nm.

2.5 Cell culture

Human Keratinocytes cells (HaCat) was cultivated in Eagle medium modified by Dulbecco (DMEM - LGC Biotecnologia, Cotia, Brazil), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, New York, USA) and kept in cell culture flasks (TPP, Switzerland), incubated in an oven at 37 °C, in atmospheric humidity and with 5% CO2. For the cytotoxicity evaluation, 4 x 10^4 cells / mL were transferred to 96-well microplate wells, where they were cultivated in DMEM medium supplemented with FBS and incubated for 24 hours for cell adhesion. Afterwards, the cells were submitted to the application of 10 different concentrations of *P. granatum* extract, with 5 minutes of contact. The cell culture medium was used as control. After the treatments, the cells were sent to the MTT colorimetric test.

2.6 Cell viability by MTT colorimetric test

The test was performed with 100 µL/well of the MTT solution, followed by incubation in the dark for 1 hour at 37 ºC. After this period, the solution was removed and 100 µL of DMSO was added, then, the plate was again incubated at 37ºC for 10 minutes. Next, the plate was placed in the Shaker under constant agitation for 10 minutes. After this, the plate was read at 570 nm and the obtained optical densities was

converted using the formula: % viability = (OD treated group x 100) / (Average OD control group)

2.7 Statistical analysis

The obtained data were submitted no normality tests and then were analysed by the ANOVA test complemented with the Tukey test, with a 5% ($p \leq 0.05$) significance level. Data were analysed using GraphPad Prism 5.0 software.

3 Results and Discussion

3.1 CIM and CMM

P. granatum L. extract showed antimicrobial effect on all the tested *A. baumannii* carbapenem-resistant strains. The MIC was 3.12 mg / mL for all the tested strains except of H571 strain which was 0.78 mg / mL. The CMM was 3.12 mg / mL for all the tested strains except of the strains H652, H656 and H571 which was 12.5, 6.12 and 0.78 mg / mL, respectively.

3.2 Antibiofilm action

The extract showed a significant reduction in biofilm metabolic action for all the tested *A. baumannii* carbapenem-resistant strains presenting a significant difference with the control group ($p \leq 0.05\%$). The concentration of 50 mg / mL was more effective than the concentrations of 12.5 and 25 mg / mL (Table 1) and presented a significant difference of the control group.

Table 1 – Reduction of metabolic action of *A. baumannii*, after 5 min in contact with pomegranate extract

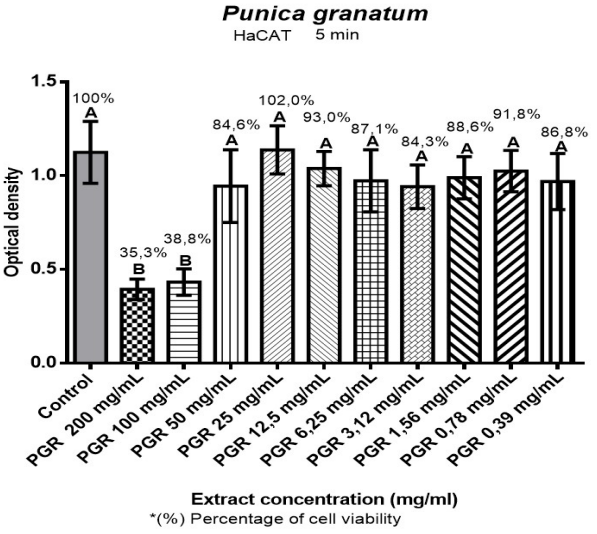
***A. baumannii* strains**	**Reduction of metabolic action (%)**		
Extract concentration	**12.5 mg / mL**	**25 mg / mL**	**50 mg / mL**
H557	12.3	0.58	87.8
H571	33.8	44.0	94.9
H652	21.1	31.8	74.6
H718	31.6	31.0	71.4
H6004	33.8	44.0	94.9
H6005	37.0	43.3	13.0
H6006	33.2	16.6	50
H656	39.9	44.6	84.5

Legend: Average % of reduction action showed by monotypic biofilms of clinical multidrug-resistant *A. baumannii* strains.
Source: Resource data.

3.3 Cell viability by MTT colorimetric test

P. granatum L. extract applied for 5 min on human keratinocytes (HaCat) promoted cell viability of 35.3%, 38.8%, 84.6%, 102.0%, 93.0%, 87.1 %, 84.3%, 88.6%, 91.8% and 86.8% after contact with the concentrations of 200; 100; 50; 25; 12.5; 6.25; 3.12; 1.56; 0.78; 0.39 mg / mL, respectively. All the tested concentrations were biocompatible as there was no significant difference with the control group except of the concentrations of 200 and 100 mg / mL (Figure.1).

Figure 1 – Cell viability of *P. granatum* extract applied for 5 min on human keratinocytes (HaCat)



Legend: Letters A and B represent the difference or statistical similarity among the groups.
Source: Resource data.

The use of phytotherapy and herbal medicine is gaining an increased space to be used to combat multi-drug resistant microorganisms and to treat infections[18,20,27-29] as the use of antibiotic agents increases the resistance of microorganisms, making it difficult to cease the disease[3,30]. Moreover, some antibiotics, such as aminoglycoside antibiotics induces nephrotoxicity[31]. In this study, *P. granatum L* extract was analysed in order to determine its efficacy against carbapenem-resistant *A. baumannii* strains.

P. granatum L ethanol extract at 250 µg / mL presented antimicrobial effect over *A. baumannii,* and it enhanced significantly the action of novobiocin at 1 µg / ml. Although the papers present different methodologies, the study by Phatthalung et al.[23] demonstrates that low concentrations of pomegranate extract demonstrate antimicrobial action. In the present study, the *P. granatum L* glycolic extract was effective over the same microorganism at lower concentrations (12.5, 25 and 50 mg / mL).

In another study, Nozohour et al.[32], evaluated the *P. granatum* ethanolic extract on strains of *P. aeruginosa* and *S. aureus*. MIC values of the extracts peel and pomegranate seeds were 12.5 and 25.0 mg / mL, respectively. In addition, the MMC was 25.0 and 50 mg / mL, respectively. In all the bacterial isolates studied, the MIC and MMC values for the pomegranate seed extract were significantly higher than those of the pomegranate peel extract. The study by Nozohour et al. corroborates the results of the present study, where it is possible to verify the antimicrobial potential of the pomegranate extending over other pathogens. However, an important point to be highlighted is the difference among the types of extracts applied, as they used alcoholic extract of the seeds and pomegranate peel while the present work applied the fruit glycolic extract. The alcohol formulation was

avoided due to its toxicity.

Furthermore, other studies evaluated the effect of the *P. granatum* over other microorganisms including *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa* and it was effective in promoting increased the pathogen antimicrobial sensitivity[24,25].

The antimicrobial action of *P. granatum* extract on planktonic cultures can also be observed in the work by Khormali et al.[33], where the researchers elaborated a Dy2O3/ ZnO-Au nanocompound using pomegranate extract, applying on standard strains of *Staphylococcus aureus*, *Proteus mirabilis* and *A. baumanni* using different concentrations ranging from 5 to 80 mg / mL. Despite the difficulty comparing among the respective studies, where Khormali et al.[33] used nanocomposites materials with pomegranate extract while the present work uses the promegranate glycolic extract, it is possible to verify that the plant species demonstrates microbial action acting even on *A. baumanni* resistant to carbapenems as demonstrated in the present study.

In this study, the pomegranate extract showed a significant reduction in metabolic action in biofilm for all the strains, with statistically different results from control group ($p \leq 0.05\%$). Comparing the results of reduced metabolic action in biofilms from clinical strains of *A. baumannii*, it is possible to observe that the pomegranate extract showed reductions ranging from 0.58% to 94.9%, showing a great reduction for seven clinical strains. Considering the results of the microdilution test, it seems that the pomegranate extract has a great microbicide effect for planktonic cells by *A. baumannii* carbapenem-resistant. In a similar study, Liberato et al., evaluated the antibiofilm action and toxicity of *Persea americana* extract against *A. baumannii.* The results showed an inhibitory and bactericidal effect against the multidrug-resistant strains tested. The lowest MIC value was at 3.12 mg / mL and all of the tested concentrations (12.5, 25 and 50 mg / mL) did not demonstrate toxicity in *Galleria mellonella* larvae[20].

In the evaluation, the cytotoxicity of pomegranate natural extract in keratinocyte culture (HaCat), in an assay of cellular mitochondrial action by the MTT method, within 5 minutes, promoted a significant cell viability in all the tested concentrations in relation to the control group, a result that can also be observed in the statistical analysis. Eight of the tested concentrations showed averages above 80% similar to the control group. It seems that these concentrations are adequate and did not show cytotoxicity. This finding may be corroborated by a study published in 2020, by Acquadro et al. In order to find out if the pomegranate extract antiviral effect against Zika virus was due to its toxicity, Acquadro et al. [34], evaluated its cell viability on Vero cells by MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetra-zolium, inner salt) assay. The authors used *P. granatum* leaf extract, *P. granatum* autumn sample and *P. granatum* summer samples. The studied concentrations were 10 mg/mL and 2.5 mg/mL and the evaluation occurred after 24h and 72h. The results demonstrated that the tested concentrations were not cytotoxic to Vero cells.

The cytotoxicity results corroborate the clinical applicability of the pomegranate extract, since the administration of glycolic extracts occurs topically and the present work demonstrated that 8 concentrations of the extract did not promote toxicity on human keratinocytes (HaCat), promoting superior cell viability at 80%, in addition to promoting the fight against planktonic cultures and biofilms of *A. baumanni* resistant to carbapenemics, a pathogen of great clinical importance that routinely affects patients initially with skin infections and later progresses to septcemia.

4 Conclusion

The pomegranate extract presented antimicrobial and antibiofilm action against the multidrug-resistant clinical strains of *Acinetobacter baumanni* and is biocompatible.

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