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Avaliação antimicrobiana do extrato seco obtido da casca e arilo da *Punica granatum*

Antimicrobial evaluation of *Punica granatum* peel and aril dry extract

Evaluación antimicrobiana de la cáscara de *Punica granatum* y extracto seco de arilo

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Resumo

As plantas medicinais têm sido usadas pelos homens no tratamento de doenças desde os tempos antigos. O Brasil é um país com grande diversidade biológica e cultural que mantém o consumo de fitoterápicos como prática popular e há um crescente interesse mundial em produtos derivados de plantas. A *Punica granatum*, popularmente conhecida como romã, é amplamente distribuída no Brasil e, segundo estudos, apresenta um bom potencial anti-inflamatório e antimicrobiano devido à presença de compostos fenólicos (taninos, terpenóides, entre outros) em sua composição. Por esse motivo, o presente estudo teve como objetivo avaliar o potencial antimicrobiano *in vitro* do extrato seco da casca de frutos de *P. granatum* pela técnica de perfuração em ágar contra cepas bacterianas de *Staphylococcus aureus* e *Escherichia coli* e do fungo *Candida albicans*. Os poços de inoculação dos extratos de *P. granatum* produziram halos de inibição consideráveis, especialmente em concentrações mais altas (5, 2,5, 1,25 e 0,62 mg). Foi demonstrada atividade antibiótica bacteriana e também fúngica. Esse desempenho da planta já era esperado, levando em consideração os diversos estudos publicados sobre o assunto. Concluímos que *P. granatum* pode ser usado na produção de novos fitoterápicos antibacterianos.

Palavras-chave: *Punica granatum*; Planta medicinal; Atividade antimicrobiana.

Abstract

Medicinal plants have been used by men in the treatment of diseases since ancient times. Brazil is a country with great biological and cultural diversity that keeps the consumption of herbal medicines as popular practice and there is a growing worldwide interest in plant-derived products. *Punica granatum*, popularly known as pomegranate, is widely distributed in Brazil and according to studies, it presents a good anti-inflammatory and antimicrobial potential due to the presence of phenolic compounds (tannins, terpenoids among others) in their composition. For this reason, the present study aimed to evaluate the *in vitro* antimicrobial potential of the dry extract of the peel of fruits of *P. granatum* by drilling technique in agar against bacterial strains of *Staphylococcus aureus* and *Escherichia coli* and the fungus *Candida albicans*. The inoculation wells of *P. granatum* extracts produced considerable inhibition halos, especially in higher concentrations (5, 2.5, 1.25 and 0.62 mg). Bacterial and also fungal antibiotic activity was demonstrated. This performance of the plant was already expected taking into account the various studies published on the matter. We concluded that *P. granatum* can be used in the production of new antibacterial phytotherapics.

Key words: *Punica granatum*; Medicinal plant; Antimicrobial activity.

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Resumen

Los hombres han usado plantas medicinales en el tratamiento de enfermedades desde la antigüedad. Brasil es un país con gran diversidad biológica y cultural que mantiene el consumo de hierbas medicinales como práctica popular y existe un creciente interés mundial en productos derivados de plantas. *Punica granatum*, popularmente conocida como granada, está ampliamente distribuida en Brasil y, según los estudios, presenta un buen potencial antiinflamatorio y antimicrobiano debido a la presencia de compuestos fenólicos (taninos, terpenoides, entre otros) en su composición. Por esta razón, el presente estudio tuvo como objetivo evaluar el potencial antimicrobiano in vitro del extracto seco de la cáscara de las frutas de *P. granatum* mediante la técnica de perforación en agar contra cepas bacterianas de *Staphylococcus aureus* y *Escherichia coli* y el hongo *Candida albicans*. Los pozos de inoculación de los extractos de P. granatum produjeron halos de inhibición considerables, especialmente en concentraciones más altas (5, 2.5, 1.25 y 0.62 mg). Se demostró actividad antibiótica bacteriana y también fúngica. Este rendimiento de la planta ya se esperaba teniendo en cuenta los diversos estudios publicados al respecto. Llegamos a la conclusión de que *P. granatum* se puede utilizar en la producción de nuevos fitoterapias antibacterianas.

Palabras clave: *Punica granatum*; Planta medicinal; Actividad antimicrobiana.

1. Introduction

Medicinal plants are the oldest "weapons" used by man in the treatment of diseases of all kinds. The use of plants in the prevention and/or cure of diseases is a habit that has always existed in the history of mankind. Thus, phytotherapy is seen as an option in the search for therapeutic solutions used mainly by the low-income population (Moraes & Santana, 2001).

Brazil is a country with great biological and cultural diversity and, therefore, counts on a considerable accumulation of traditional knowledge and technologies, among which the vast body of knowledge on the management and use of medicinal plants stands out. Several cultural groups resort to plants as a therapeutic resource, and in recent years, their use has intensified as an alternative or complement to traditional drug treatments (Dorigoni et al., 2001).

Punica granatum (Pomegranate) belongs to the *Punicaceae* Family and is an ornamental and also medicinal plant (Silva, 2001). It is a woody, branched shrub, native from the region of Iran up to the Himalayas in northwest India. It has been cultivated for a long time throughout the Mediterranean region of Asia, America, Africa and Europe. It presents small, hard, shiny and membranous leaves, red-orange flowers arranged at the ends of the branches, giving rise to spherical fruits, with many layered seeds which are enveloped in pulpy arils (Lorenzi & Souza, 2001; Ferreira, 2004).

Pharmacological properties of *Punica granatum* L. have been extensively described, resulting in their indication for several uses, including immunomodulation, arteriosclerosis, bacterial infection, fungal infection, parasitic infection and periodontal disease (Longtin, 2003). In Brazil, the pomegranate and its parts (leaves, stem bark and fruits) are used in the treatment of throat infections, hoarseness and fever, as antiseptic and antiviral in inflammatory processes of the oral mucosa and against genital herpes (Lansky & Newmann, 2007).

Among the constituents present in the plant are flavonoids (apigenin and narigenin), anthocyanins, tannins (gallic and ellagic acids), alkaloids, ascorbic acid, conjugated fatty acids (punic acid) and ursolic acid (Lansky & Newmann, 2007). Pomegranate peel extract is also rich in polyphenols, which have proven strong antiseptic effect and also antibacterial activity against gram-negative and gram-positive bacteria (Negi & Jayaprakasha, 2003).

The objective of this work was to evaluate the *in vitro* antimicrobial potential of the *P. granatum* dry extract using the agar drilling technique against bacterial strains, *Staphylococcus aureus* and *Escherichia coli*, and the fungal species *Candida albicans.*

2. Material and Methods

2.1 Plant Material

Plant material was collected in the community garden of the Primavera Leste county, Teresina, Piauí, Brazil. The voucher is deposited in the Graziela Barroso Herbarium - UFPI under registration number 30120.

2.2 Extract preparation

2.2.1 Hydroalcoholic extract preparation

The extraction process was carried out by maceration with 70% (v/v) hydroalcoholic solution (water:ethanol) of ethanol at room temperature. A proportion of 20% (w/v) of plant material (peel + mesocarp of the fruit) was used in this process. The suspension was maintained for 72 hours in polyethylene terephthalate vial protected from light with daily stirring. After 72 hours, the suspension was vacuum filtered using qualitative filter paper, obtaining this way the fluid extract of *Punica granatum.*

2.2.2 Dry extract preparation by Spray Dryer

Spray drying involves the atomization of a liquid containing solids in solution, suspension or emulsion in an apparatus suitable for this technique. The liquid or paste is atomized using a centrifugal or high pressure system, where the atomized droplets immediately come into contact with a flow of hot air. The rapid evaporation of the water allows the temperature of particles to be kept low, so that the high temperature of the drying air does not affect the product much (Silva et al., 2012). In this research, the drying process of the extracts was carried out using the spray drying technique in Mini Spray Dryer Büchi B-290. The extractive solution under homogenization on magnetic stirrer was subjected to the apparatus under the following conditions: inlet temperature of 170°C, flow rate of 10% (approximately 3 mL/min), pressure of 0.9 barr and aspiration in 90% of the equipment capacity.

2.2.3 Bacterial strains and Preparation of Mc Farland's standard solution 0.5

The study of antimicrobial activity of the dried extract of the fruit and aril of *Punica granatum* was used against the standard of *Staphylococcus aureus* (ATCC 25923), Escherichia coli (ATCC 25922) strains and Candida albicans (ATCC 10231) analyzed in antibiogram in Mueller-Hinton agar medium, using the agar drilling technique. The colony of the standard strain was touched with a bacteriological platinum strap properly flambéed and cooled. The methodology according to the CLSI-2013 (Clinical and Laboratory Standards Institute) was used to standardize the density of the inoculum for the test of sensitivity. The colonies were suspended in sterile saline solution (0.85% NaCl) until a turbidity compatible with the Mac Farland scale grade 0.5 ($1x10^6$ CFU / mL) was obtained.

2.2.4 Agar drilling technique

The process was carried out in triplicate, totaling 9 plates containing Mueller Hinton Agar, where 3 were used for the culture and analysis of the microbial activity of the dry extract against the fungus *C. albicans*, and 3 for each bacterium selected in this study. The plates were prepared in advance and were removed from the refrigerator until reaching room temperature. With a sterile swab, bacterial and fungal inocula were evenly distributed on the agar surface until exhaustion; then the plates were allowed to stand at room temperature for approximately 3 minutes.

In the agar drilling technique, the solid culture medium is the removed with the aid of cylinders 6-8 mm in diameter to form wells, in which the substances to be analyzed can be applied (Ostrosky et al., 2008).

Then, 8 wells with 6 mm diameter were made in each plate and 50 ml of the solution previously diluted in the dry extract were placed in different concentrations and compared with a positive (gentamicin (GEN), oxacillin (OXA)) and negative control (dimethylsulfoxide (DMSO)).

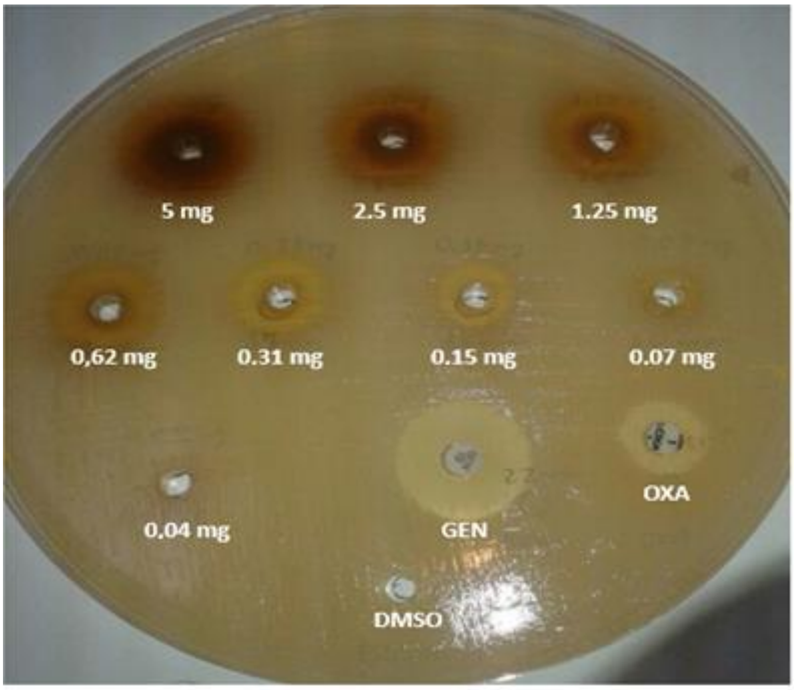
3. Results and Discussion

P. granatum is a plant rich in bioactive compounds, and some authors have already studied its antimicrobial potential for hydroethanolic extracts (Moreira et al., 2014). The aim of this work was to evaluate the dry extract of *Punica granatum* in relation to the antimicrobial potential of already established antibiotics.

According to CLSI (2013), antimicrobial sensitivity tests are indicated for any organism that causes an infectious process and requires antimicrobial therapy, when it is possible to predict the sensitivity of this organism, and can be used to verify the *in vitro* sensitivity of the microorganisms before antimicrobial agents.

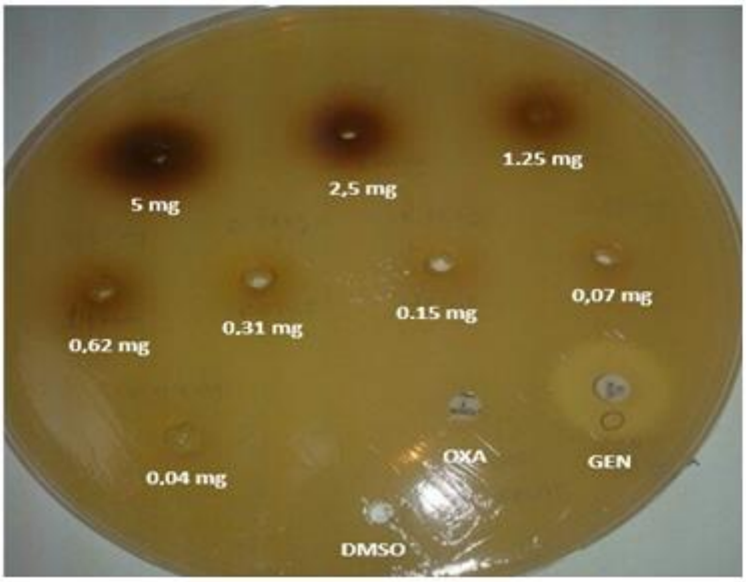
The antimicrobial activity of the dried extract of *Punica granatum* was evaluated by the drilling method in MH agar (Figures 1, 2 and 3) because it is an easy and inexpensive technique.

Figure 1. Antimicrobial activity against *Staphylococcus aureus* in different concentrations of *Punica granatum* extract compared to gentamicin (GEN), oxacillin (OXA) and dimethylsulfoxide (DMSO).



Source: Author's collection.

Figure 2. Antimicrobial activity against *Escherichia coli* in different concentrations of *Punica granatum* extract compared to gentamicin (GEN), oxacillin (OXA) and dimethylsulfoxide (DMSO).



Source: Author's collection.

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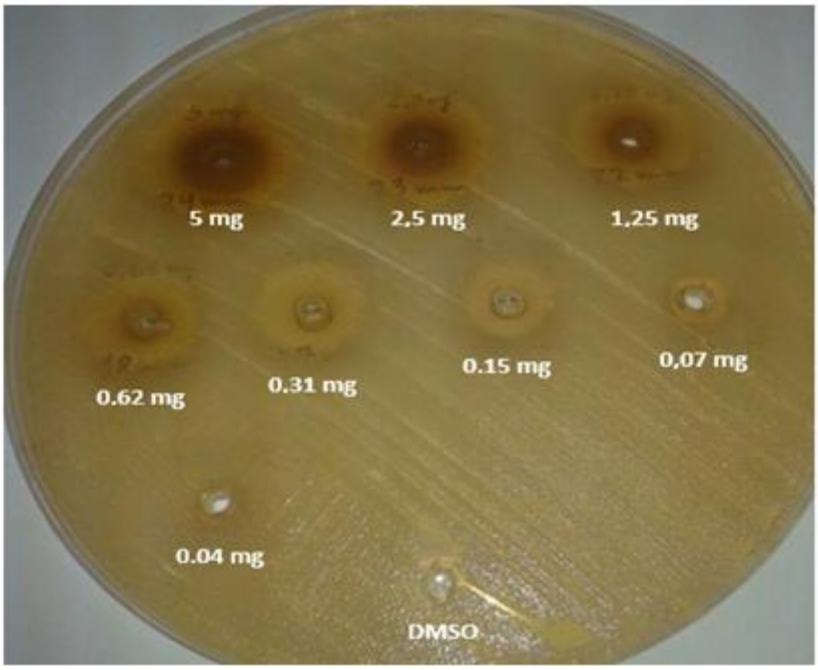
Figure 3. Antimicrobial activity against *Candida albicans* in different concentrations of *Punica granatum* extract in comparison to dimethylsulfoxide (DMSO).



Source: Author's collection.

In the case of *Staphylococcus aureus* and *Escherichia coli*, oxacillin and gentamicin were tested as controls, and in the fungus *Candida albicans*, only DMSO was used as control. According to Brunton (2011), oxacillin is part of the subgroup of isoxazolyl penicillins and is, in this case, an agent with remarkable resistance to penicillinase cleavage. According to the bulletin of the ANVISA portal, oxacillin is indicated only in the treatment of infections caused by penicillinase-producing staphylococci, which are sensitive to the drug, resulting in a small antimicrobial spectrum due to their specificity of action. However, several studies, such as Silva et al (2012) have demonstrated that oxacillin has reduced activity against previously sensitive strains, even in some *S. aureus* strains whose infection was its main field of action. A similar result can be seen in Figure 1, where the OXA well showed a lower inhibition halo against *S. aureus* than that caused by the GEN well and even lower than the wells at concentrations 5, 2.5 and 1.25 mg of the *P. granatum* extract. Moreover, some colonies of *S. aureus* were able to grow in the diameter of the halo. Therefore, considering the total inhibition in the halo region as a parameter, we chose to classify the activity as presenting no inhibition.

On the other hand, as the aminoglycoside gentamicin is a complex antibiotic (gentamycins C1, C2, C1a, C2a, C2b), it has a broad antibacterial spectrum and is indicated for the treatment of serious infections caused by Gram-negative microorganisms (Lourenço, 2006). As shown in Table 1, 10 μg of gentamicin are capable of promoting inhibition halos above 15 mm in both *Staphylococcus* spp. and *Enterobacteriaceae* strains. Thus, the results observed in Figures 1 and 2, where the GEN inoculation wells in both cases showed considerable inhibition halos are fully plausible.

Antimicrobial activity is not restricted only to bacteria, because traditionally pomegranate-based dentifrices are among the main allies in the fight against fungal infections of the oral cavity. Menezes et. al., (2008) report that a *P. granatum* gel was used in the treatment of candidiasis associated with prosthetic stomatitis, observing the efficacy of *P. granatum* as a topical antifungal agent for yeasts of the genus *Candida*. Other studies that evaluated the effect of pomegranate extract on periodontal treatment concluded that clinical signs of chronic periodontitis were significantly reduced after treatment. The synergism of fluconazole with pomegranate peel extract against *Candida albicans* was confirmed in vitro by Endo et al., (2010). This combination represents a perspective for the development of products for candidiasis.

In the three agar plates, the inoculation wells related to the concentrations of the *P. granatum* extract produced considerable inhibition halos, especially in those with higher concentrations (5, 2.5, 1.25 and 0.62 mg), which demonstrates not only bacterial but also fungal antibiotic activity (Table 1).

Table 1. Inhibition Halos (mm) obtained by the drilling method in agar used in the analysis of the hydroalcoholic extract of the *P. granatum* peel at different concentrations.

Extract concentration	***S. aureus***				***E. coli***				***C. albicans***			
	P1	P2	P3	AM	P1	P2	P3	AM	P1	P2	P3	AM
5.0	22.00	21.00	22.00	21.67	20.00	19.00	19.00	19.33	24.00	24.00	24.00	24.00
2.5	21.00	21.00	20.00	20.67	17.00	17.00	18.00	17.33	23.00	24.00	22.00	23.00
1.25	20.00	21.00	20.00	20.33	16.00	15.00	16.00	15.67	22.00	22.00	21.00	21.67
0.62	19.00	19.00	19.00	19.00	14.00	14.00	14.00	14.00	18.00	19.00	19.00	18.67
0.31	17.00	16.00	16.00	16.33	-	-	-	n.d.	17.00	17.00	17.00	17.00
0.15	15.00	16.00	15.00	15.33				n.d.	15.00	17.00	15.00	15.67
0.07	10.00	8.00	9.00	9.00				n.d.	10.00	8.00	11.00	9.67
0.04	-	-	-	n.d.				n.d.	-	-	-	n.d.
Antibiotics												
Oxalicin	-	-	-	-	-	-	-	-	n.d.	n.d.	n.d.	n.d.
Gentamicin	22.00	23.00	22.00	22.33	21.00	19.00	19.00	19.67	n.d.	n.d.	n.d.	n.d.
Control												
DMSO	w.i.	w.i.	w.i.	w.i.	w.i.	w.i.	w.i.	w.i.	w.i.	w.i.	w.i.	w.i.

Legend - P1: Plate 1; P2: Plate 2; P3: Plate 3; MA: Arithmetic mean; n.d .: not determined; (-): resistant; w.i .: without inhibition.
Source: author's collection.

Costa (2002), offers detailed botanical, ethno-pharmacological and pharmacological information about *P. granatum*. According to the author, pomegranate trees have been used for a long time because of their anthelmintic activity, mainly against *Taenia solium* worm, because their compounds act in the nervous system of helminths causing paralysis. Such ethno-pharmacological knowledge already indicated an anti-parasite activity that if evaluated more thoroughly could be transposed to organisms at the microbiological level. Thus, *P. granatum* has gained renewed evidence in the last decades from *in vitro* studies on the anti-cariogenic action of tannins (compounds present in the species). These studies have demonstrated the *in vitro* anti-bacterial and anti-adherent action of extracts from innumerable plants, among them the pomegranate, on Gram-positive and Gram-negative microorganisms. Moreover, *Streptococcus mitis*, *Streptococcus mutans* and *Streptococcus sanguis* showed sensitivity to pomegranate extract with respect to growth, presenting inhibition halos up to 20 mm in diameter (Pereira et al., 2005).

According to Costa (2002), the pomegranate pericarp (peel) and the flowers contain very small amounts of alkaloids, but are rich in tannins (therefore used as astringents) and anthocyanins: cyanidin-3-glucoside, cyanidin- 3.5-diglucoside and delphinidin-3-glucoside. In its composition has been also identified gallic and ellagic acids, resin, starch and ash. Alkaloids occur in percentages varying from 0.3 to 0.9%, with some exceptions. Thus, pharmacological studies and characterizations indicate that not only one but a group of constituents of *P. granatum* are considered responsible for its pharmacological properties, among which alkaloids (peletierin, isopeletierin, metilpeletierin), tannins, phenolic compounds (anthocyanins, quercetin, phenolic acids) and flavonoids (Silva et al., 2013).

P. granatum stem contains up to 20% of a hydrolyzable tannin, in part combined with alkaloids, forming insoluble combinations that justify their pharmacological properties. Thus, besides the tannins, the antimicrobial properties of *P. granatum* are attributed also to the alkaloid dl-pelletierine and especially when these are combined. The two natural isomers of the alkaloid are oily, volatile liquids with water vapor, with alkaline properties, which in the air are converted into resin, soluble in water, ether and chloroform, and forming crystalline salts with acids. Another alkaloid, the pseudopelletierine or N-methylgranatonin, has been identified in *P. granatum* extracts, which has a different molecular structure but is easily related to pelletierine; it is a strong, crystalline and fixed tertiary base, soluble in water, ether and chloroform (Costa, 2002).

The main constituents identified in *P. granatum*, among which there are at least two with recognized antimicrobial properties (tannins and alkaloids) and the inhibition halos in the plates containing *Staphylococcus aureus* (Figure 1), *Escherichia coli* (Figure 2) and *Candida albicans* (Figure 3) confirm the evidence regarding this promising pharmacological activity of pomegranate, especially when comparing the results of the different concentrations of the extract with the antibiotics known to be potent inhibitors of bacterial strains analyzed in this study, such as gentamicin (Table 1). Therefore, it is possible to affirm that pomegranate-based products for bactericidal and fungicidal purpose are efficient and more in-depth and robust studies with patients infected by staphylococci resistant to oxacillin are necessary at the moment, as well as studies on the benefits from *P. granatum* based products.

4. Final Considerations

The results obtained in the present work demonstrate that the dry extract obtained through *Spray Dryer* of the peel and aril of the fruit of *Punica granatum* presented antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* bacteria, and against the fungus *Candida albicans*. The performance of the analyzed plant converged with the results of previous experiments published in scientific literature. We concluded that *P. granatum* can be used in the production of new antibacterial phytotherapic products.

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