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Evaluation of the antifungal capacity of hydroalcoholic extract of pomegranate peel (*Punica granatum L*) and application as a natural preservative in bread.

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Abstract

Natural extracts have bioactive compounds concentrated in leaves, peels, and seeds. In this research, pomegranate peel extract was tested as a natural conservant for bread. The hydroalcoholic extract of pomegranate peel was obtained by dehydrating the peels, powdering them, and then performing a maceration in 70% ethanol; high-performance liquid chromatography characterized the extract to quantify punicalagin. Fungal strains (e.g., *Penicillium paneum*, *Penicillium citrinum*, *Cladosporium* spp., and *Aspergillus chevalieri*) isolated from deteriorated commercial bread were used to determine antifungal activity via minimum inhibitory concentration/ fungicidal concentration, mycelial growth, and spore inhibition tests, with morphological effects assessed by scanning electron microscopy. Additionally, the preservative potential of the extract was evaluated by applying different concentrations to bread samples and monitoring microbial growth over time. The extract demonstrated potent antifungal activity, with minimum inhibitory concentrations of 2.50% for *Penicillium paneum*, 1.25% for *Penicillium citrinum*, and 5.0% for *Aspergillus chevalieri*, while *Cladosporium* strains showed resistance. In bread preservation tests, pomegranate peel extract at concentrations of 0.625–2.50% maintained low mold and yeast counts up to the 25th day, demonstrating comparable performance to synthetic preservatives. The hydroalcoholic extract of pomegranate has shown efficiency as a preservative and natural alternative for combating and preventing fungal infections in bakery products.

Keywords: pomegranate extracts; natural preservative; *Aspergillus*; *Penicillium*; *Cladosporium*.

Practical Application: Obtaining, characterizing, and applying a natural preservative from pomegranate peel.

1 INTRODUCTION

According to Živančev et al. (2024), contamination of food products by fungi and their toxic metabolites results in approximately a quarter of qualitative and quantitative losses in agricultural food production worldwide. Recent studies using plant-derived biopesticides and "green techniques" for fungal control have shown promising results, such as alternatives to the use of synthetic antifungals. The use of essential oils and plant extracts against the main genera of fungi associated with spoilage and production of mycotoxins in foods, such as *Aspergillus* spp. (Naseer et al., 2014), *Alternaria* spp. (Castro et al., 2017), *Cladosporium* spp. (Prakash et al., 2012), *Fusarium* spp. (Elsherbiny et al., 2016; Osorio et al., 2010), and *Penicillium* spp. (Quaglia et al., 2016).

Industries in the food sector are showing a growing demand for natural preservatives, which has prompted investigations into their application for preserving food in general, especially perishables such as meat. This market has been demanding natural and preservative-free antioxidants, as well as synthetic additives. Therefore, the search for natural preservatives has increased considerably. Pomegranate extracts (*Punica granatum* L.) have demonstrated antioxidant and antimicrobial properties with significant potential for use in food and pharmaceutical products (Grillo et al., 2023).

Pagliarulo et al. (2016) evaluated the antimicrobial activity of pomegranate peel extract (PPE) against the growth of *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, and *Saccharomyces cerevisiae* and the antioxidant effect on the stability of sunflower oil. The results showed significant antimicrobial effects and high antioxidant activity, indicating the potential for using this extract as a multifunctional preservative in food products.

Few studies have investigated the effect of pomegranate extracts on baked products, a widely consumed food item with a short shelf life. Thus, the objective of this work was to investigate the *in vitro* antifungal capacity of the crude hydroalcoholic extract of pomegranate peels and to evaluate its preservative action in bread.

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1.1 Relevance of the work

Due to the great demand for natural preservatives and antioxidants that the food and beverage market presents, this work can make an important contribution, showing a viable alternative to using pomegranate extract for this purpose. The article shows in detail the obtainment and characterization of this extract, as well as its application in the conservation of a short shelf-life food that is widely consumed in Brazil and worldwide. The crude pomegranate peel extract (PPE), rich in punicalagin, inhibited fungal growth, causing damage to the hyphae development. It also considerably reduced the viability of the spores produced when we applied 1 and 5% of PPE. In the *in vivo* test, small concentrations of the extract provided the bread with a shelf life equivalent to the action of the synthetic preservative used commercially. Thus, these results support a possible use of natural extract as an effective alternative to control spoilage in foods and meet the demand for agents rich in antioxidant and antimicrobial compounds.

2 MATERIAL AND METHODS

2.1 Obtention of the hydroalcoholic extract of pomegranate peels (P. granatum L)

The pomegranates (cultivar California) were harvested in the production unit, at Northeast of Paraná (23°29′05.4″S 52°03′07.1″W).

The peels were manually separated from the arils, washed in deionized water, and dehydrated in a laboratory drying oven for 48 h at 50°C. Afterward, the peels were crushed in a knife mill until obtaining a 60-mesh flour and preserved under vacuum at −18°C.

PPE was obtained by applying the maceration technique. The peel flour was homogenized with a 70% hydroalcoholic solution at a 1:10 (m/v) ratio for 24 h.

The supernatant was centrifuged (Centribio) at 3,500 rpm for 15 min, vacuum-filtered in a Büchner funnel with a 0.5 µm filter (Zeta Plus), and rotary evaporated (Büchi RE 120) at 50°C until dry.

2.2 Extract characterization

An HPLC system (Dionex UltiMate 3000, Thermo Scientific, Bannockburn, IL, USA) equipped with a quaternary analytical pump (model LPG-3400SD Dionex, Bannockburn, IL, USA) and an autosampler (model WPS-3000TSL Dionex Analytical, Bannockburn, IL, USA) with a photodiode array detector (model DAD-3000 Dionex, Bannockburn, IL, USA) and adjustment of $\lambda = 254$ nm for the quantification of punicalagin.

Gradient elution was performed on a Nucleosil C18 column (Macherey-Nagel; Germany) with 250 × 4.6 mm i.d. and 5 µm particle size and a mobile phase composed of two solvents, solvent A (H_2O with 0.1% HOAc) and solvent B (MeOH).

The pomegranate hydroalcoholic extract was filtered through a 0.22 µm nylon syringe and injected at 5 µL into the column. A calibration curve was used with a punicalagin analytical standard (Sigma-Aldrich), which, in solution, shows the mixture of the two anomers (α and β punicalagin) with an external standard calibration curve (R_2 [α punicalagin] > 0.9934 and R_2 [β punicalagin] > 0.9933). Gradient elution was performed on a Nucleosil C18 column (Macherey-Nagel; Germany) with 250 × 4.6 mm i.d. and 5 µm particle size and a mobile phase composed of two solvents, solvent A (H_2O with 0.1% HOAc) and solvent B (MeOH).

2.3 Fungal isolates

For microbiological evaluations, strains *of Penicillium paneum, Penicillium citrinum, Cladosporium oxysporum, Cladosporium subliforme, and Aspergillus chevalieri* isolated from deteriorated commercial bread were used and identified through molecular techniques and morphological analysis.

Fungal isolation was carried out with 10 samples of commercial bread from different brands, purchased at local commerce. It was stored at room temperature until visually detectable deterioration began to occur. Afterward, fungal isolation was conducted in potato dextrose agar (PDA). The colonies were grouped according to their morphology and transferred to Petri dishes containing agar-agar and then incubated at 20°C for 5 days.

The extraction of fungal genomic deoxyribonucleic acid (DNA) using the PowerSoil DNA Isolation kit (MoBio Laboratories, Inc.) was performed according to the manufacturer's instructions, except for the initial phase, as fungi were grown in PDA, and approximately 200 mg of mycelium was used for extraction. DNA integrity was checked on a 1% agarose gel.

2.4 Minimum inhibitory concentration and minimum fungicidal concentration tests

For the minimum inhibitory concentration (MIC) tests, the macrodilution method was employed, following the standards of the M38-A technique recommended by the National Committee for Clinical Laboratory Standards, with adaptations for filamentous fungi and natural products (Capoci et al., 2015).

2.5 Mycelial growth verification

According to the technique described by Cao et al. (2024) with modifications, the PPE was dissolved directly in malt extract agar (MEA) and PDA medium to obtain concentrations of 1 and 5% of the extract. Each strain was subcultured in MEA for 4 days at 25°C.

2.6 Spore viability and sporulation inhibition tests

Inhibition of sporulation and spore viability was performed in triplicate (with colonies inoculated in PDA) as proposed by Cao et al. (2024) with some changes.

2.7 Scanning electron microscopy

Penicillium paneum, *P. citrinum*, *C. oxysporum*, *C. subliforme*, and *A. chevalieri* were cultivated in a control medium

(PDA) and a medium containing 1 and 5% of PPE, incubated at 28°C for 7 days. We cut the mycelial discs and fixed them with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, then treated them with poly-l-lysine (Sigma-Aldrich). Sequentially, samples were dehydrated in a descending series of ethanol concentrations and then dried through a CO_2 critical point. They were metalized with gold and examined using a Shimadzu SS-550 scanning electron microscope (Endo et al., 2010).

2.8 Valuation of the conservation of bread with pomegranate peel extract added

The conditions for the application of PPE were guided by the values of MIC and the indication of the manufacturer of the commercial anti-mold liquid used as a control (healthy bread anti-mold, containing INS420, INS422, INS200, and grain alcohol: 60–75 g of healthy bread per liter of cereal alcohol or 96°GL ethyl alcohol). The bread used in this test was manufactured by a local industrial bread manufacturer, which formulates its products without preservatives.

Synthetic preservative and the PPE solutions diluted in grain alcohol at the working concentrations (0.4 g of solution) were sprayed over the entire surface of the bread. Each sample had 450 g, industrially produced and preservative-free, according to the groups: group 1—proof in white with a spray of pure grain alcohol, undiluted; group 2—control with sprinkles of preservative diluted in grain alcohol as indicated by the manufacturer; and groups 3, 4, 5, 6, and 7 with concentrations of 0.625, 1.25, 2.50, 5.0, and 10.0% of the extract, respectively.

The conservation evaluation was performed every 5 days of storage through visual analysis of the products and detection of mold and yeast count (triplicate).

2.9 Statistics

The data obtained were statistically analyzed using Statistica Single User 13.2. Mean values, standard deviation, maximum, and minimum for all quantitative variables were calculated. After verifying the non-normality of the data by the Shapiro-Wilk test, non-parametric tests were chosen. The Kruskal–Wallis test was performed to compare three or more groups and verify possible statistical differences between the evaluated groups, followed by the Dunn test (for multiple comparisons). A 5% significance level was adopted for the tests, i.e., significant comparisons where $p < .05$.

3 RESULTS AND DISCUSSION

3.1 Chemical composition of hydroalcoholic pomegranate peel extract

According to Akhtar et al. (2015), the skin that contains the highest concentration of high molecular weight polyphenols, including punicalagin, comes from all anatomical parts of the pomegranate. These substances are found only in pomegranates. They are part of the family of ellagitannins, including tannins (punicalin and ellagic acid). All of them have good solubility in water.

The chromatogram (not shown) revealed the majority presence of punicalagin as α and β anomers. Concentrations of 0.20 μg/μL of α-punicalagin and 0.27 μg/μL of β-punicalagin were found in the crude extract. That is equivalent to 6.7 and 9.0% of α and β punicalagin (3 μg/μL), respectively.

Pagliarulo et al. (2016) obtained the two isomers of punicalagin as the main components of alcoholic extract (50%) from the peels. On the other hand, Rongai et al. (2016) mostly quantified punicalagin and ellagic acid in the aqueous extract of pomegranate peels.

Unlike the cited works, Elsherbiny et al. (2016) identified chlorogenic acid and catechin as the predominant polyphenols in the methanol extract of pomegranate peels.

3.2 Evaluation of antifungal (minimum inhibitory concentration) and fungistatic (minimum fungicidal concentration) capacity

The hydroalcoholic PPE showed significant values for MIC and minimum fungicidal concentration (MFC), as well as expressive inhibition of mycelial growth for the strains tested (Table 1). Except for *C. oxysporum* and *C. subliforme*, which showed resistance to the extract in the MIC and MFC tests, *P. paneum* and *A. chevalieri* have the same concentration values for inhibition and fungicidal action: 2.50 and 5.0%, respectively.

Penicillium citrinum was the most sensitive strain and showed a higher concentration of extract (5%) for fungicidal action. For fungistatic action, 1%. Similar results were found for other genera and plant extracts in Elsherbiny et al. (2016) in *Fusarium sambucinum,* reaching the MIC with 2% of PPE % and MFC with 12% of it.

3.3 Evaluation of inhibition of mycelial growth, sporulation, and spore germination

A concentration-dependent inhibition of mycelial growth was observed for *P. citrinum*, *C. oxysporum*, and *C. subliforme*. *P.*

Table 1. Minimum inhibitory concentration and minimum fungicidal concentration of the hydroalcoholic extract of pomegranate on *Penicillium paneum*, *Penicillium citrinum*, *Cladosporium oxysporum*, *Cladosporium subliforme,* and *Aspergillus chevalieri*.

Strains	1-Pp	2-Pc	3-Co	4-Cs	5-Ac
MIC (%)	2.50	1.25	ND	ND	5.0
MFC (%)	2.50	2.50	ND	ND	5.0

MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration; ND: there was no inhibitory or fungicidal action; 1-Pp: *Penicillium paneum*; 2-Pc: *Penicillium citrinum*; 3-Co: *Cladosporium oxysporum*; 4-Cs: *Cladosporium subliforme*; 5-Ac: *Aspergillus chevalieri.*

citrinum was the most susceptible fungus to inhibition (Table 1). In the control group, the *C. oxysporium* showed lower levels compared to *P. paneum* (p = .0119). At 1% of PPE, the *P. paneum* showed higher levels compared to *P. citrinum* (p = .0062), and the same occurred at 5% of PPE (p = .0031).

Although relevant and specific studies were not found for the same strains discussed here, microorganisms of the same genera were evaluated in the presence of PPE, as reported by Naseer et al. (2014), with inhibition of *Aspergillus flavus* observed at 0.01% alcoholic PPE.

Eldiasty et al. (2014) showed 70% of mycelial inhibition of *Penicillium* sp. and 31% of *A. niger* in the presence of 10 and 20% alcoholic PPE, respectively. At 30% of PPE, there was total inhibition of the development of both strains. Aqueous extract of *P. granatum* L. peels, cultivar Hicaznar, and three other pomegranate genotypes inhibited the growth of *Aspergillus parasiticus* mycelium from 30.90 to 38.63% at a concentration of 0.32% of extract (Nawaz et al., 2025).

Glazer et al. (2012) used an aqueous extract of pomegranate peel at concentrations of 25 and 55% for antifungal tests with isolated fruit strains. They found a stimulus to the mycelial growth of *Penicillium expansum* and *Botrytis cinerea* in the presence of the extract. They did not observe any effect along with the development of *Penicillium digitatum*. However, there was inhibition of the growth rates of *Alternaria alternata*, *Stemphylium botryosum*, and *Fusarium* spp. in the presence of the aqueous extract of peels. Similarly, Tanveer et al. (2015) suggested that the antimicrobial activity associated with phenolic compounds present in plants and their extracts may be related to their ability to interact with the chemical structure of cell membranes in microorganisms. This could result in changes in permeability, a reduction in cytoplasmic content, protein precipitation, and consequent cell damage and inhibition of development.

In addition to phenolic compounds, other groups of substances also comprise the category of antimicrobials, including phenolic acids, quinones, saponins, flavonoids, tannins, coumarins, terpenoids, and alkaloids (Shi et al., 2024).

Changes in resistance reflect physiological adaptation to an adverse environmental condition, epigenetic changes, or stable genetic changes, depending on the nature of the drug, the duration of exposure, and the organism (LeBlanc et al., 2020).

In the spore counting of the 1% PPE group, the level presented in the 2-Pc strain was statistically higher than in the 4-Cs one (p = .0132). On the other hand, in the 5% of the PPE group, strain 2-Pc was higher when compared to the 5-Ac strain (p = .0091), as shown in Table 1. *A. chevalieri* was the only strain that showed reduced sporulation at 1% of the extract concentration. However, like the others, they had a stimulus at the maximum concentration of extract applied in the test.

Although they did not exhibit significant reductions in sporulation, the germination power of some strains was affected by the ethanol extract present in the pomegranate peels (Table 1).

Spores cultivated in PDA and germinated in media containing 1 and 5% PPE significantly impaired *C. oxysporum* (84% of inhibition) and *C. subliforme* (90%) development with 5% of the extract. *P. citrinum* (95% inhibition) and *A. chevalieri* (93.30%) showed decreased germination at 1% PPE, maintaining the same value for PPE 5%. *P. paneum* was the most resistant strain to the test (Figure 1).

Regarding the spores cultivated in a medium containing PPE at 1 and 5% and germinated in PDA, *C. subliforme* decreased its germination capacity as the concentration of PPE increased (73% of inhibition at 5% of PPE). *C. oxysporum* (63% of inhibition) and *P. citrinum* (95%) were significantly reduced to 1%. *P. paneum* once again showed resistance to the test, and *A. chevalieri* showed total germination inhibition at both concentrations.

In sporulation tests performed by Glazer et al. (2012), *S. botryosum* spores showed 74% inhibition of germination with the crude extract. There was an inhibition of 85% with 5 and 10% of a fractionated methanolic extract rich in punicalagin and 63% with the aforementioned extract at 30%.

Nicosia et al. (2016) obtained similar results, showing 100% of germination inhibition in *B. cinerea*, 91% for *P. digitatum*, and 82.7% for *P. expansum* when using an aqueous extract of pomegranate peels at a concentration of 1.2% (12

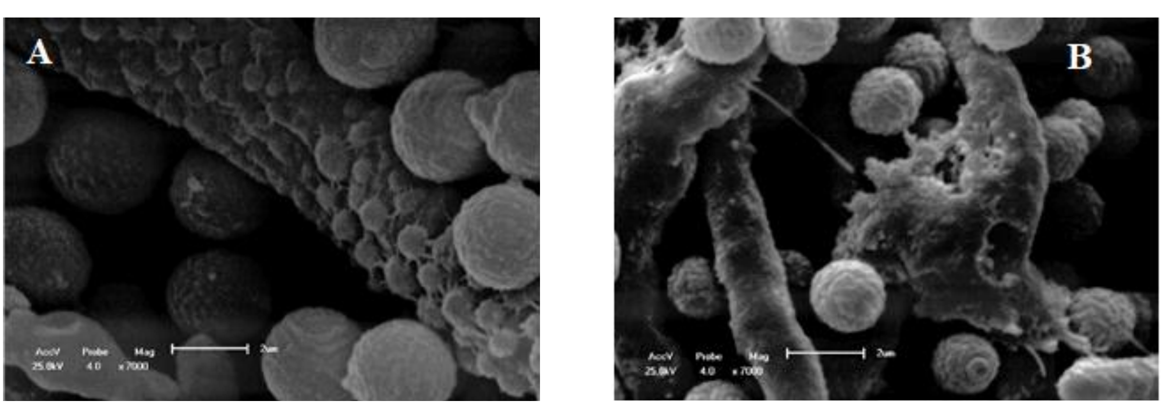

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Figure 1. **(A)** *Penicillium paneum* at 5% extract concentration. **(B)** Cell damage in *Penicillium citrinum* at 5% concentration of pomegranate peel extract.

g/L) with a high concentration of phenolics with punicalagin as its predominant component. Jiménez-Moreno et al. (2019) employed aqueous and methanolic extractions on leaves, seeds, and pomegranate peels. They obtained 90% more mycelial inhibition in the methanolic extract. Methanolic extracts of peel and seed did not show a significant difference in the values of inhibition of spore germination. *Penicillium italicum* had been inhibited at 17.86%, *Rhizopus stolonifer* at 30.29%, and *B. cinerea* at 30.92%.

3.4 Scanning electron microscopy

All strains used in this study, grown in PDA without any interference from the addition of PPE, showed no visible structural changes. *P. paneum* was the fungus that suffered the least interference from the PPE on its growth and germination and did not exhibit any structural anomalies. On the contrary, *P. citrinum* exhibited ruptures in its hyphae and extravasation of cellular content, as shown in Figure 1.

It is possible to visualize structural disruptions and morphological changes, as well as the possible formation of adhesive mucilage in *Cladosporium* strains. These changes possibly affected the development and structural characteristics of the isolates. Manso et al. (2013) found significant structural changes and inhibition of sporulation in *A. flavus* exposed to 10, 20, and 30 μL of cinnamon essential oil.

Castro et al. (2017) identified structural alterations of *A. alternata* in the presence of *Cinnamomum zeylanicum*, *Eugenia caryophyllus*, and *Cymbopogon flexuosus* extracts.

3.5 Evaluation of the conservation of bread

The initial count of molds and yeasts, representing time 0, corresponding to the day of bread manufacturing and the beginning of the experiment, showed a value lower than 1×10^2 CFU/g of bread for all groups studied. During the storage time, the counting remained constant until the 25th day in all tests for the yeast group.

Regarding the visual evaluation, the products remained free from superficial alterations due to fungal growth until the 20th day for all tests. On the 25th day of storage, the white, control, 5, and 10% PPE groups exhibited mold development, and by the 30th day, all tests were contaminated with mold.

There was no significant difference between the tests in the mold counting until the 20th day. The bread treated with 0.625, 1.25, and 2.5% PPE remained with a count lower than 1×10^2 CFU/g until the 25th day of storage.

The addition of 0.1% PPE provided a 2-week increase in shelf life for the chicken meat product (Kanatt et al., 2010). Meat pate added with PPE also had good refrigeration conservation results (Hayrapetyan et al., 2012).

4 CONCLUSIONS

The crude PPE, rich in punicalagin, inhibited fungal growth, causing damage to the hyphae development. It also considerably reduced the viability of the spores produced when applied at 1 and 5% PPE concentrations. In the *in vivo* test, small concentrations of the extract provided the bread with a shelf life equivalent to that of the synthetic preservative used commercially. Thus, these results support a possible use of natural extracts as an effective alternative to control spoilage in foods and meet the demand for agents rich in antioxidant and antimicrobial compounds.

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