BIOSCIENCE JOURNAL

ANTIFUNGAL ACTIVITY OF *Punica granatum* LINN EXTRACTS AGAINST *Malassezia pachydermatis*

Sophia Aparecida Morro CHAMILETE[1], Leopoldo Sussumu MATSUMOTO[1], Fernanda DELFINO[2], Guilherme Villa PIRAJÁ[3], Regildo Márcio Gonçalves DA SILVA[4], Erika Cosendey Toledo de Mello PEIXOTO[1]

[1] Degree in Veterinary Medicine, Center of Agrarian Sciences, Universidade Estadual do Norte do Paraná, Bandeirantes, Paraná, Brazil.
[2] Médica Veterinária Autônoma, Jaú, São Paulo, Brazil.
[3] Veterinário da Clínica Veterinária e Pet Shop Dog e Cia, Marília, São Paulo, Brazil.
[4] Degree in Biological Sciences, Center of Biotechnology, Universidade Estadual Paulista, Assis, São Paulo, Brazil.

Corresponding author:
Erika Cosendey Toledo de Mello Peixoto
emellopeixoto@uenp.edu.br

How to cite: CHAMILETE, S.A.M., et al. Antifungal activity of *Punica granatum* linn extracts against *Malassezia pachydermatis*. *Bioscience Journal*. 2023, **39**, e39098. https://doi.org/10.14393/BJ-v39n0a2023-66031

Abstract

Malassezia pachydermatis causes external otitis, often affecting dogs, and control methods for this microorganism have been resistant to synthetic antifungals. Therefore, this study evaluated the antifungal activity of aqueous extracts of *Punica granatum* Linn (AEP) fruit peel dehydrated (AEPd) and *in natura* (AEPn) against *Malassezia pachydermatis*. The *M. pachydermatis* samples were from the Microbiology Laboratory of the State University of Northern Paraná (UENP), PR, Brazil. The strains were identified and replicated after inoculation in the Sabouraud dextrose medium. Subsequently, the *P. granatum* extract was obtained through different extraction methods: cold, water bath, decoction, and infusion. Each test was run fivefold at 10, 20, 30, 40, and 50% after 10, 20, and 30 minutes. The sensitivity of isolates was determined with the Kirby-Bauer disc diffusion method and indicated by an inhibition zone larger than 15 mm. The results were evaluated with a 2x3x6 factorial study design, ANOVA, and Tukey's test at 5% significance. AEPn showed antifungal activity on *M. pachydermatis* strains, and AEPd did not present an inhibitory influence at any concentration and time. Extraction by decoction was the most efficient, followed by water bath, cold, and infusion. The extracts at a 50% concentration showed the best results, but all other doses determined an inhibition zone larger than 15 mm. Thus, AEP showed a significant therapeutic potential for controlling *M. pachydermatis*.

Keywords: External otitis. Medicinal plants. Pomegranate.

1. Introduction

Using medicinal plants in veterinary therapy is beneficial because they are safe, easily acquired, and inexpensive (Vianna et al. 2016). *Punica granatum* Linn (flavonoids, phenolic acids, and tannins) has been extensively used due to its phytocomplex, presenting good adaptation to the body and reducing side effects and adverse reactions (Santos et al. 2019).

The pomegranate tree belongs to the Punicaceae family and is characterized as a woody shrub, branched with multiple stems, growing approximately 1.8 - 4.6 m in height. It has glossy deciduous leaves

measuring 7 cm in length, on average, and orange-red trumpet-shaped flowers with raised petals of approximately 5 cm in length (Gunjan et al. 2012).

Abdu et al. (2020) found that extracts prepared from pomegranate peel showed antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, and antifungal activity against *Candida parapsilosis*. Al-Defiery et al. (2021) found that the *Punica granatum* peel extract was effective against Gram-positive and Gram-negative pathogenic bacteria, with potential use for treating infections caused by antibiotic-resistant microorganisms.

External otitis is common in dogs and can be unilateral or bilateral (Bajwa 2019). It is characterized by an external acoustic meatus inflammatory process caused by the association of predisposing, primary, and perpetuating factors (Chan et al. 2018). Recurrent inflammations can evolve into severe glandular changes, fibrosis, stenosis, and calcification of the external ear canal, allowing an acute infection to become chronic (Bajwa 2019).

Malassezia pachydermatis is a fungus that causes external otitis in dogs, presenting discomfort and itching. Magalhães et al. (2017) found that 60.9% of animals diagnosed with external otitis showed positive cytological results for yeasts, and all samples identified *Malassezia* spp. That is a perpetuating factor of external otitis because it exacerbates the inflammatory process and maintains the disease after eliminating the primary factor (Sharma et al. 2017). Therefore, diagnosing the condition is essential.

However, indiscriminate antimicrobial use has contributed to the selection of resilient microorganisms. Thus, *M. pachydermatis* has been resistant to synthetic antifungals, such as ketoconazole, itraconazole, fluconazole, and amphotericin B (Deegan et al. 2019). This research evaluated *in vitro* the antifungal activity of *P. granatum* extract (AEP) against *M. pachydermatis*.

2. Material and Methods

Experiment 1

Preparation of the aqueous extract of Punica granatum *Linn*

Aqueous extracts were made from *P. granatum* Linn fruit peel *in natura* (AEPn) and dehydrated (AEPd). The fruits were washed under running water and peeled manually.

To obtain the AEPn, 100 g of fresh fruit peel was added to 200 mL of distilled water. After boiling, it was filtered in a sterile cotton filter, and the evaporated water was added until obtaining a 50% concentration (mass/volume). The resulting solution originated the following dilutions: 10, 20, 30, and 40%.

To obtain the AEPd, the fruit peel was dried in a forced ventilation oven (DeLeo®, Porto Alegre, Brazil) at 65°C and 1.5 meters per second (m/s) until reaching constant weight. Then, the AEPd was processed as described for AEPn.

Determining the antifungal activity of the aqueous extract of pomegranate

The *M. pachydermatis* samples originated from the Microbiology Laboratory of the State University of Northern Paraná (UENP), PR, Brazil. The samples were inoculated in Petri dishes containing Sabouraud dextrose agar medium and incubated in a bacteriological incubator (DeLeo®, Porto Alegre, Brazil) for 72 consecutive hours at 37°C.

The macromorphology of *M. pachydermatis* colonies was identified according to the technique by Sidrim and Moreira (1999), characterizing colonies with soft or friable texture and creamy-yellow coloration. Subsequently, microscopic identification and Gram staining were performed for confirmation.

An *M. pachydermatis* suspension at $1x10^6$ CFU mL^{-1} of cell concentration was obtained by adjusting the solution to the McFarland scale 6 and evaluating it through light spectrophotometry with absorbance readings in a spectrophotometer at wavelengths of 600 nm and 0.6 to 0.69 nm (National Committee for Clinical Laboratory Standards [NCCLS] 2002).

After defining cell concentration, successive dilutions were made in saline solution until obtaining 10^3 CFU mL^{-1}. One milliliter of sterile distilled water was added to the control treatment for each milliliter of the resulting suspension. AEPn and AEPd were added to the other treatments at 10, 20, 30, 40, and 50% concentrations, leaving the extracts working for 10, 20, and 30 minutes. After these respective times, 50 µL of each treatment was inoculated in Petri dishes containing Sabouraud dextrose agar using the pour plate technique and incubated at 37°C for 72 hours in a bacteriological incubator (DeLeo®, Porto Alegre, Brazil). The results were obtained fivefold.

The CFU mL^{-1} values were determined by counting the colonies aided by a colony counter and transforming the results into log CFU mL^{-1}. The study design was 2x3x6 factorial, and the data were submitted to the analysis of variance, followed by Tukey's test at a 5% significance.

Experiment 2

Preparation of the aqueous extract of* Punica granatum *Linn

After obtaining the aqueous solution from the fruit peel at a 50% concentration, different extraction methods were analyzed: water bath (AEPbm), decoction (AEPd), infusion (AEPi), cold (AEPc), and negative control (sterile saline solution).

The first aqueous peel extract was submitted to a water bath at 60°C for 60 minutes using a filter funnel with hydrophilic cotton. The volume was measured, stored in a sterile amber glass vial, and frozen at -20°C (Electrolux® Super Freezer Model DC4, Stockholm, Sweden). This procedure was also applied to obtain the aqueous peel extract by decoction. Distilled water was heated to a boil and received the crushed fruit peel, boiling this mixture for 10 minutes.

Extraction by infusion occurred by adding the plant material to boiling water, remaining for 60 minutes. As for the cold aqueous extract, the plant material was mixed in water at room temperature and luminosity for 24 hours. All extracts were filtered through a funnel with absorbent cotton and diluted at 40, 30, 20, and 10% (w/v) concentrations.

Determining the antifungal activity of the aqueous extract of pomegranate

In a unidirectional flow chamber (Grupo Veco®, Campinas, Brazil), disc filters (8 mm) were impregnated fivefold with 40 µL of each extract, dried for 60 minutes, and fixed on plates with PDA (Potato-Dextrose-Agar) medium previously seeded with *M. pachydermatis*. Incubation occurred in a bacteriological incubator at 37°C for 72 hours (DeLeo®, Porto Alegre, Brazil).

The results showing *M. pachydermatis* inhibition zones larger than 15 mm (Cardoso et al. 2019) were considered sensitive to the treatments. These sensitive concentrations were submitted to ANOVA and Tukey's test at a 5% probability, using the STATISTICA - StatSoft, 2007 program.

3. Results

Experiment 1

The *P. granatum* Linn extracts *in natura* (AEPn) showed significant inhibitory action at 40 and 50% concentrations only after 30 minutes (Table 1). It was impossible to verify antimicrobial activity in any of the other concentrations. Similarly, the dehydrated extract did not allow the assessment of inhibitory influence for any concentration and time evaluated in the present study (Figure 1).

Experiment 2

Regarding the concentrations, the more concentrated the extract, the larger the formed inhibition halo. Figure 2 shows the distribution of arithmetic means (in millimeters, mm) in these zones, according to

AEPbm, AEPd, AEPi, and AEPc concentrations in *Malassezia pachydermatis*. As for extraction type, the larger inhibition zones (mm) occurred for AEPd, AEPbm, AEPi, and AEPc consecutively (Figure 3).

The analysis of variance (ANOVA) showed an interaction between water bath, decoction, infusion, and cold extractions and the evaluated concentrations (10, 20, 30, 40, and 50%) (p=0.000081).

Table 1. Analytical mean growth of *Malassezia pachydermatis* (log CFU mL^{-1}) subjected to the aqueous extracts of *Punica granatum* L. *in natura* (AEPn) and dehydrated (AEPd) at 0, 10, 20, 30, 40, and 50% concentrations for 10, 20, and 30 minutes of treatment.

Time	AEPn					
	0%	10%	20%	30%	40%	50%
10	6.55 Aa	6.46 Aa	6.44 Aa	6.46 Aa	6.44 Aa	6.22 Aa
20	6.55 Aa	6.42 Aa	6.55 Aa	6.53 Aa	6.65 Aa	6.29 Aa
30	6.55 Aa	6.26 Aa	6.14 Aa	4.27 Bab	2.18 Bb	1.99 Bb
	AEPd					
10	6.55 Aa	6.68 Aa	6.60 Aa	6.44 Aa	6.33 Aa	6.14 Aa
20	6.55 Aa	6.70 Aa	6.63 Aa	6.35 Aa	6.40 Aa	6.06 Aa
30	6.55 Aa	6.26 Aa	6.56 Aa	6.24 Aa	6.21 Aa	5.89 Aa

Means followed by the same capital letter in the column and the same lowercase letter in the row do not differ by Tukey's test at p<0.05. AEPn: Aqueous extract of *Punica granatum* Linn *in natura*, AEPd: Aqueous extract of *Punica granatum* Linn dehydrated (Pirajá 2014).

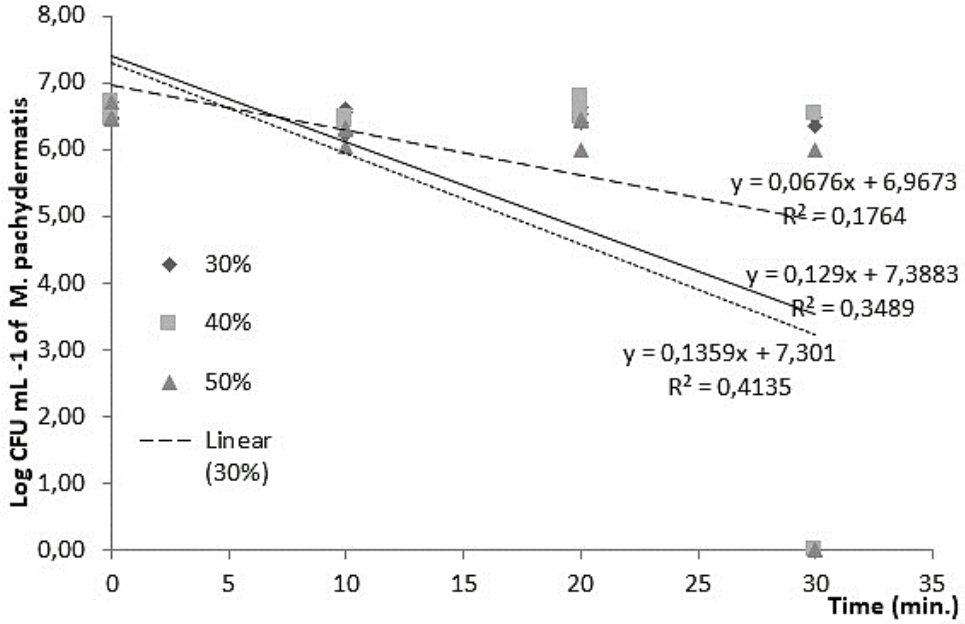

Figure 1. Correlation of the effect of exposure time (minutes) to pomegranate extract at 30, 40 and 50% concentrations on *Malassezia pachydermatis* growth (Log CFU mL^{-1}) (Pirajá 2014).

4. Discussion

Experiment 1

Madugula et al. (2017) verified that the minimum fungicidal concentration of the *Punica granatum* peel extract was 10 µl/mL, with 50% inhibition in *Candida* sp. They also stated that pomegranate peel extract showed antifungal efficacy analogous to clotrimazole. Another study found that 80% of pomegranate peel extract showed antifungal activity against dermatophytes, such as *Trichophyton mentagrophyte* 1, *Trichophyton verrucosum*, *Trichophyton terrestrial*, *Trichophyton mentagrophytes* 2, and *Microsporum boullardii* (Easa et al. 2018).

Bassiri-Jahromi et al. (2018) demonstrated that pomegranate peel extract at 500 µg/mL concentration was effective against oral fungi after five days. However, all doses (500, 250, and 125 µg/mL) showed a 100% cure against oral candidiasis after 15 days, concluding that the longer the action time, the higher the product effectiveness. These data corroborate the present study, which demonstrates a positive

correlation (r^2 = 0.41) between antimicrobial efficacy and the longer action time (30 minutes) of the AEPn extract (Figure 1).

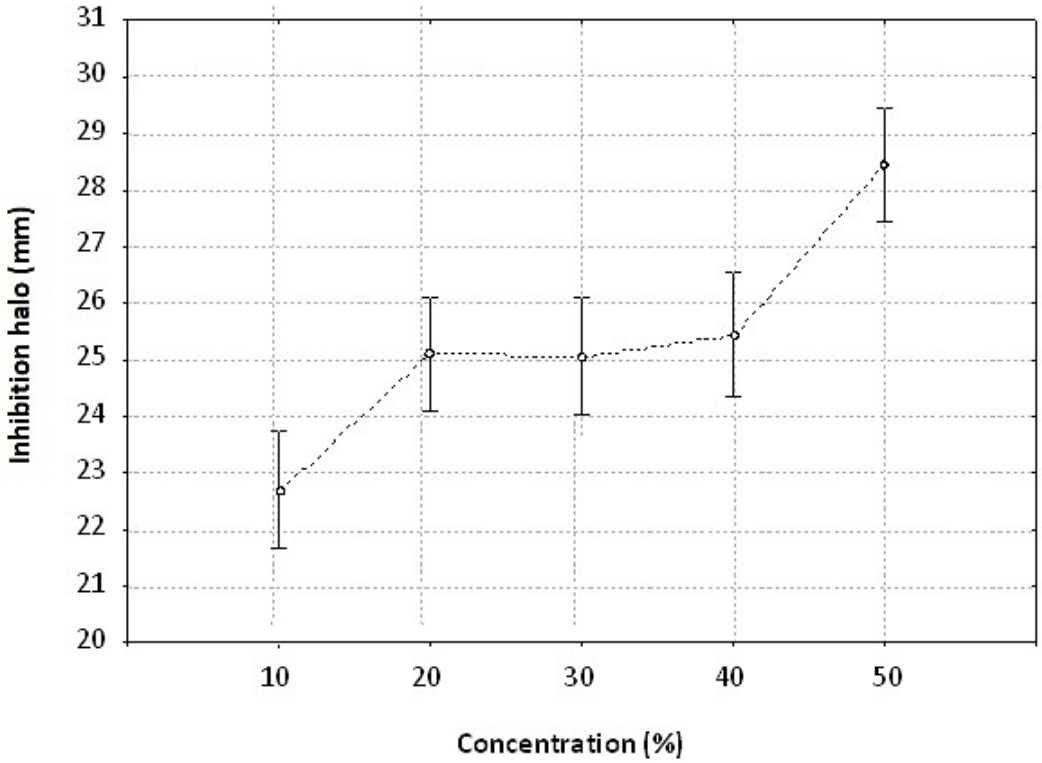

Figure 2. Distribution of arithmetic means of the inhibition halos (mm) according to the concentration of *Punica granatum* Linn. Barck extracts in *Malassezia pachydermatis* (Delfino 2014).

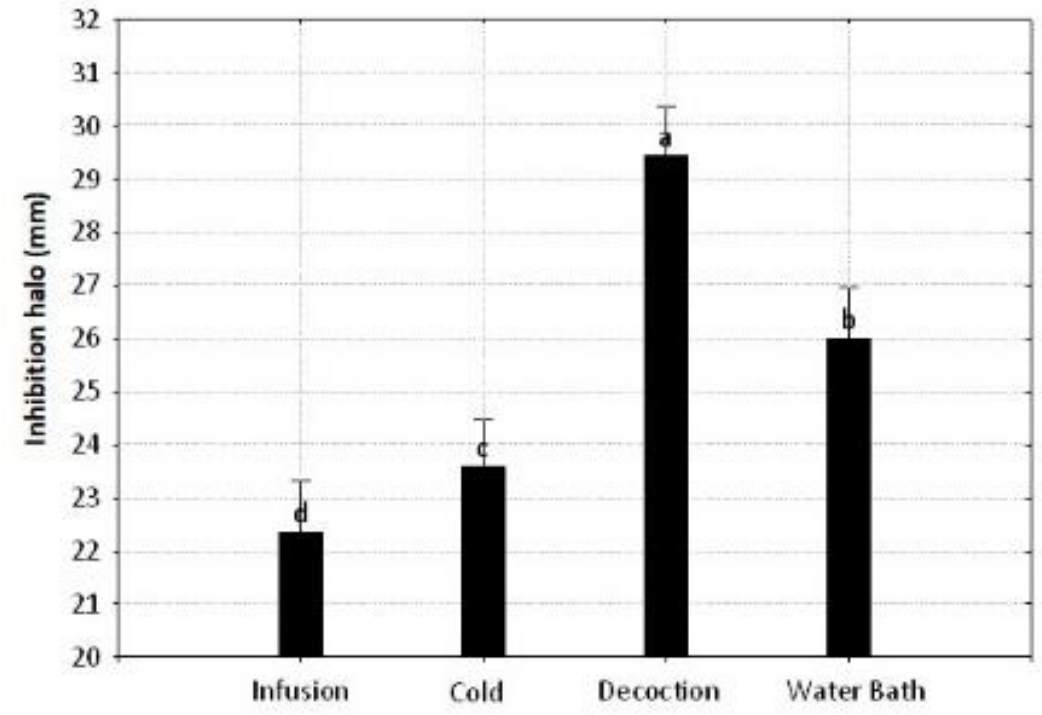

Figure 3. Distribution of the arithmetic means of the inhibition halos (mm) in *Malassezia pachydermatis* according to extraction type: infusion, cold, decoction and water beth (Delfino 2014).

Regarding the raw material used, the fruit peels were dehydrated to obtain dry weight values. However, the heating process harmed the extract components' biological activity, similar to Venturoso et al. (2010).

Franzener et al. (2003) reinforce the possibility of secondary metabolites of thermosensitive plants. These authors evaluated the antifungal activity of camphor extract and found that the autoclaved extract did not inhibit *Bipolaris sorokiniana* spore germination.

The present study used heating to obtain the *P. granatum* Linn extract, unlike Pereira et al. (2006), who did not recommend this procedure because the material is rich in polyphenols with easy structural modifications (Kharchoufi et al. 2018).

Experiment 2

Pomegranate peel is rich in tannin, saponin, quinine, terpenoids, steroids, flavonoids, phenols, alkaloids, glycosides, cardiac glycosides, coumarin, anthocyanin, and betacyanin. These secondary metabolites are responsible for pharmacological and therapeutic activity. However, aspects such as genetic differences, environmental factors, and extraction techniques should be considered to explain the qualitative and quantitative differences among the bioactive constituents of pomegranate (Pirzadeh et al. 2021).

Pomegranate peel compounds have been effective against Gram-negative bacteria (*Escherichia coli*, *Salmonella* sp, *Pseudomonas aeruginosa*, *P. putida*, *Enterobacter aerogenes,* and *Klebsiella pneumonia*) and Gram-positive bacteria (*Bacillus subtilis*, *Listeria innocua*, *L. monocytogenes*, and *Staphylococcus aureus*) (Wafa et al. 2017; Kharchoufi et al. 2018).

Turrini et al. (2020) corroborated the present study by verifying that the aqueous extract of pomegranate peel by traditional decoction was not only easy to obtain but also showed higher values of ellagic acid, total phenolics, radical scavenging capacity, and total anthocyanin content compared to a new extraction technique using pulsed ultrasound. Leite et al. (2014) also demonstrated that the aqueous extract of pomegranate peel by decoction at 60, 80, and 100 mg/mL concentrations promoted higher inhibition effects against *Pseudomonas aeruginosa* than chlorhexidine.

In the present study, the aqueous extract of pomegranate peel by decoction presented the best result, differing from Ghosh et al. (2019), whose infusion method showed higher antioxidant activity, simplicity, and cost-effectiveness. The solubility difference of plant extract components varies according to the solvent and its preparation. Therefore, water seems to be the best solvent for extracting phytochemical elements from pomegranate peel (Khedoudja et al. 2020). Savikin et al. (2018) also observed that pomegranate aqueous extracts showed more punicalin and punicalagin than those prepared with ethanol, petroleum ether, ethyl acetate, and butanol.

5. Conclusions

The aqueous extract of *Punica granatum* Linn *in natura* showed antifungal activity in the *Malassezia pachydermatis* strain at 40 and 50% concentrations after 30 minutes of action. Regarding the different extraction methods, decoction showed the best results. These findings may represent a significant therapeutic potential for controlling canine otitis, allowing to minimize the use of synthetic antifungals.

Authors' Contributions: CHAMILETE, S.M.: conception and design, data acquisition, analysis, and interpretation, and article drafting; MELLO-PEIXOTO, E.C.T.: data analysis and interpretation, article drafting, and critical review of relevant intellectual content; MATSUMOTO, L.S.: data analysis and interpretation and critical review of relevant intellectual content; PIRAJÁ, G.V.: data acquisition, analysis, and interpretation and article drafting; DELFINO, F.: data acquisition, analysis, and interpretation and article drafting; SILVA, R.M.G: critical review of relevant intellectual content. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

Acknowledgments: To the National Council for Scientific and Technological Development – Brazil (CNPq), the Araucária Foundation, and the State University of Northern Paraná (UENP).

References

ABDU, O.H., SAEED, A.A. and FDHEL, T.A. Polyphenols/flavonoids analysis and antimicrobial activity in pomegranate peel extracts. *Electronic Journal of University of Aden for Basic and Applied Sciences*. 2020, **1**(1), 14-19.https://doi.org/10.47372/ejua-ba.2020.1.4

AL-DEFIERY, M.E. Antimicrobial activity of garlic and Pomegranate Peel extracts against some pathogenic bacteria. *IOP Conference Series: Earth and Environmental Science*. 2021, **722**(1), 012017.https://doi.org/10.1088/1755-1315/722/1/012017

BAJWA, J. Canine otitis externa — Treatment and complications. *The Canadian Veterinary Journal*. 2019, **60**(1), 97-99.

BASSIRI-JAHROMI, S., et al. *In Vivo* Comparative Evaluation of the Pomegranate (*Punica granatum*) Peel Extract as an Alternative Agent to Nystatin against Oral Candidiasis. *Iranian Journal of Medical Sciences*. 2018, **43**(3), 296-304.

CARDOSO, R.C., et al. Potencial Antimicrobiano do Óleo da Folha de *Eucalyptus urograndis* Frente *Staphylococcus aureus*. *Revista Multidisciplinar e de Psicologia*. 2019, **13**(43), 989-1002.http://idonline.emnuvens.com.br/id

CHAN, W.Y., et al. *In vitro* antimicrobial activity of monensin against common clinical isolates associated with canine otitis externa. *Comparative Immunology, Microbiology and Infectious Diseases*. 2018, **57**, 34-38.

DEEGAN, K.R., et al. Susceptibility of *Malassezia pachydermatis* Clinical Isolates to Allopathic Antifungals and Brazilian Red, Green, and Brown Propolis Extracts. *Frontiers in Veterinary Science*. 2019, **6**, 460.https://doi.org/10.3389/fvets.2019.00460

EASA, S.M.H. Antifungal Activities of Some Plant Extracts against Pathogenic Fungi. *Egyptian Academic Journal of Biological Sciences*. 2018, **10**(1), 1-19.

FRANZENER, G., et al. Atividade antifúngica e indução de resistência em trigo a *Bipolaris sorokiniana* por *Artemisia camphorata*. *Acta Scientiarum Agronomy*. 2003, **25**(2), 503-507.https://doi.org/10.4025/actasciagron.v25i2.2124

GHOSH, S., et al. Comparison of different aqueous extraction methods for optimum extraction of polyphenols and *in vitro* anti-oxidant activity from pomegranate peel. *Journal of Pharmacognosy and Phytochemistry*. 2019, **8**(3), 42-347. www.phytojournal.com

GUNJAN, J., et al. Antioxidant activity of various parts of *Puniga granatum*: A Review. *Journal of Drug Delivery & Therapeutics.* 2012, **2** (6), 138-141.http://jddtonline.info

KHARCHOUFI, S., et al. Antimicrobial and antioxidant features of 'Gabsi' pomegranate peel extracts. *Industrial Crops & Products*. 2018, **111**, 345–352.https://doi.org/10.1016/j.indcrop.2017.10.037

KHEDOUDJA, K., et al. Demonstration of the antifungal activity of the aqueous macerated extract of pomegranate (*Punica granatum* Linn.) bark against *Rhodotorula* sp. *International Journal of Minor Fruits, Medicinal and Aromatic Plants*. 2020, **6** (2), 28- 35.

LEITE, S.J.O., et al. *In vitro* assessment of the antimicrobial effects of pomegranate (*Punica granatum* L.) peel decoction on saliva samples. *Journal of Basic and Applied Pharmaceutical Sciences*. 2014, **35** (1), 25-28.

MADUGULA, P., et al. "Rhetoric to Reality"- Efficacy of *Punica granatum* Peel Extract on Oral Candidiasis: An *In vitro* Study. *Journal of Clinical and Diagnostic Research*. 2017,**11**(1),114-117. https://doi.org/10.7860/jcdr/2017/22810.9304

MAGALHÃES, N.R., et al. Frequência de *Malassezia* spp. em cães apresentando otite externa. *Scientific Electronic Archives*. 2017, **10** (6).http://www.seasinop.com.br/revista/index.php?journal=SEA&page=article&op=view&path%5B%5D=379&path%5B%5D=pdf

NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS. Método de referência para testes de diluição em caldo para a determinação da sensibilidade a terapia antifúngica das leveduras.2002.https://www.anvisa.gov.br/servicosaude/manuais/clsi/clsi_OPAS1M38-A.pdf.

PARERA, L.A.M. The effect of ethanol extracts of red pomegranate peel (*Punica granatum* L.) on activities *Candida albicans*. *Ecology, Environment and Conservation*. 2019, **25**, 81-86.

PEREIRA, J.V., et al. Efeito antibacteriano e antiaderente *in vitro* do extrato da *Punica granatum* Linn. sobre microrganismos do biofilme dental. *Revista Brasileira de Farmacognosia*. 2006, **16** (1), 88-93.

PIRZADEH, M., et al. Pomegranate as a source of bioactive constituents: a review on their characterization, properties and applications. *Critical Reviews in Food Science and Nutrition*. 2021, **61** (6), 982–999.https://doi.org/10.1080/10408398.2020.1749825

SANTOS, E.P., et al. *Punica granatum* L. (Romã) e Atividade Antimicrobiana Contra o Biofilme Dental: uma Revisão Bibliográfica. *Ensaios e Ciência*. 2019, **23** (2), 88-93. https://doi.org/10.17921/1415-6938.2019v23n2p88-93

SAVIKIN, K., et al. Activity guided fractionation of pomegranate extract and its antioxidant, antidiabetic and antineurodegenerative properties. *Industrial Crops and Products*. 2018, **113**, 142–149.https://doi.org/10.1016/j.indcrop.2018.01.031

SHARMA, A.K., et al. Studies on Malassezia Infection in Otitis External of Dogs. *Journal of Animal Research*. 2017, **7** (1), 197-200. https://doi.org/10.5958/2277-940x.2017.00027.4

SIDRIM, J.J.C. and MOREIRA, J.L.B. Fundamentos clínicos e laboratoriais da micologia médica. Rio de Janeiro: Editora Guanabara Koogan, 1999.

TURRINI, F. Traditional Decoction and PUAE Aqueous Extracts of Pomegranate Peels as Potential Low-Cost Anti-Tyrosinase Ingredients. *Applied Sciences*. 2020, **10** (8), 2795.https://doi.org/10.3390/app10082795

VENTUROSO, L.R., et al. Influência de diferentes metodologias de esterilização sobre a atividade antifúngica de extratos aquosos de plantas medicinais. *Revista Brasileira de Plantas Medicinais*. 2010, **12** (4), 499-505.https://doi.org/10.1590/S1516-05722010000400014

VIANNA, U.R., et al. Tópicos especiais em Ciência Animal V. (1ª Edição). Alegre: Universidade Federal do Espírito Santo, 2016. Available from https://cienciasveterinarias.ufes.br/pt-br/topicos-especiais-em-ciencia-animal-teca

WAFA, B.A. Antimicrobial effect of the Tunisian Nana variety Punica granatum L. extracts against *Salmonella enterica* (serovars Kentucky and Enteritidis) isolated from chicken meat and phenolic composition of its peel extract. *International Journal of Food Microbiology*. 2017, **241**, 123-131.https://doi.org/10.1016/j.ijfoodmicro.2016.10.007

Received: 25 June 2022 | **Accepted:** 11 July 2023 | **Published:** 09 October 2023