Research, Society and Development, v. 10, n. 16, e531101623935, 2021
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v10i16.23935

Antimicrobial activity of the extract, fractions and punicalagin from the rind of the fruit of *Punica granatum* against clinical isolates from cows with mastitis

Atividade antimicrobiana do extrato, frações e punicalagina da casca do fruto de *Punica granatum* frente a isolados clínicos de vacas com mastite

Actividad antimicrobiana del extracto, fracciones y punicalagina de la cáscara del fruto de *Punica granatum* frente a aislados clínicos de vacas con mastites

Received: 11/26/2021 | Reviewed: 12/06/2021 | Accept: 12/10/2021| Published: 12/18/2021

Emannuel Ítalo Alves Campos
ORCID: https://orcid.org/0000-0002-3817-4001
Universidade Federal de Goiás, Brazil
E-mail: italoemannuel2010@gmail.com
Liliane de Sousa Silva
ORCID: https://orcid.org/0000-0001-9497-7573
Universidade Federal de Goiás, Brazil
E-mail: ssliliane@gmail.com
Sandra Alves de Sousa Garcia
ORCID: https://orcid.org/0000-0002-8103-8793
Universidade Federal de Goiás, Brazil
E-mail: sandraalvesgarcia30@gmail.com
Pollyana Guimarães de Oliveira
ORCID: https://orcid.org/0000-0002-6203-9432
Universidade Federal de Goiás, Brazil
E-mail: pollyana.bio1@gmail.com
Milton Adriano Pelli de Oliveira
ORCID: https://orcid.org/0000-0002-3444-6751
Universidade Federal de Goiás, Brazil
E-mail: mapoliv@ufg.br
Carla Afonso da Silva
ORCID: https://orcid.org/0000-0002-6648-1674
Universidade Federal de Goiás, Brazil
E-mail: carlaafonso@bol.com.br
José Realino de Paula
ORCID: https://orcid.org/0000-0002-4424-7692
Universidade Federal de Goiás, Brazil
E-mail: pjrpaula@gmail.com

Abstract
The aim of this study was to evaluate the antimicrobial activity of the extract, fractions and purified punicalagin from peel pomegranate fruit of the granada and wonderful varieties against Coagulase Positive *Staphylococcus* (CPS) and Coagulase- negative *Staphylococcus* (CNS), isolated from cows with mastitis. The pomegranate pods were dried in an oven, ground and the ethanol extract was prepared by maceration and percolation. The fractions were obtained by liquid-liquid fractionation, and the purification of punicalagin was carried out in a chromatographic column filled with Diaion® HP-20. HPLC and NMR (^{1}H and ^{13}C -Acetone-d6) were used for identification, quantification and structural elucidation of punicalagin and MIC (CLSI-M7A10) were determined for 23 clinical isolates. The punicalagin content was higher in the extract and fractions of the granada variety, reaching 81.5% in the purified punicalagin sample, which showed good antimicrobial activity against clinical isolates, with emphasis on *S. aureus* and *S. schleiferi schleiferi*, where the MIC was 31.75 µg/ml. Thus. punicalagin was defined as an important metabolite for the antimicrobial potential of *P. granatum* fruits, however, the synergism of metabolites of ethyl acetate fractions and aqueous fraction of the granada variety, had considerable importance for the good antimicrobial activity of these fractions, compared to 100 % of the CPS and CNS. Thus, the results obtained confirm the antimicrobial activity of the metabolites present in the pomegranate peel, which may support new research on pharmaceutical formulations based on *P. granatum*, as an alternative for the treatment, prevention and control of mastitis.
Keywords: Pomegranate, Granada varieties; Wonderful varieties; Minimum Inhibitory Concentration (MIC); High Performance Liquid Chromatography (HPLC).

Resumo

O objetivo deste estudo foi avaliar a atividade antimicrobiana do extrato, frações e punicalagina purificada da casca do fruto da romã, das variedades granada e *wonderful*, frente a *Staphylococcus* Coagulase Positivo (SCP) e *Staphylococcus* Coagulase Negativa (SCN), isoladas de vacas com mastite. As cascas dos frutos de romã foram secas em estufa, moídas e o extrato etanólico foi preparado por maceração e percolação. As frações foram obtidas por fracionamento líquido-líquido, e a purificação da punicalagina realizada em coluna cromatográfica preenchida com Diaion® HP-20. CLAE e RMN (^{1}H e ^{13}C -Acetona-d_6) foram utilizados para identificação, quantificação e elucidação estrutural da punicalagina e a CIM (CLSI-M7A10) foi determinada para 23 isolados clínicos. O teor de punicalagina foi maior no extrato e frações da variedade granada, chegando a 81,5% na amostra de punicalagina purificada, que apresentou boa atividade antimicrobiana frente aos isolados clínicos, com ênfase para *S. aureus* e *S. schleiferi schleiferi*, onde a CIM foi de 31,75 µg/mL. Desta forma. a punicalagina foi definida como importante metabólito para o potencial antimicrobiano dos frutos de *P. granatum* contudo, o sinergismo dos metabolitos das frações acetato de etila e fração aquosa da variedade granada, teve considerável importância para a boa atividade antimicrobiana dessas frações, frente a 100% dos SCP e SCN. Assim, os resultados obtidos confirmam a atividade antimicrobiana dos metabólitos presentes na casca da romã, podendo fundamentar novas pesquisas de formulações farmacêuticas baseadas em *P. granatum*, como alternativa para o tratamento, prevenção e controle da mastite.

Palavras-chave: Romã, Variedade granada; Variedade *wonderful*; Concentração Inibitória Mínima (CIM); Cromatografia Líquida de Alta Eficiência (CLAE).

Resumen

El objetivo de este estudio fue evaluar la actividad antimicrobiana del extracto, fracciones y punicalagina purificada de cáscara de granada del granate y *wonderful* variedades contra *Staphylococcus* Coagulase Positive (SCP) y *Staphylococcus* Coagulase Negative (SCN), aisladas de vacas con mastitis. Las vainas de granada se secaron en un horno, se molieron y el extracto de etanol se preparó mediante maceración y percolación. Las fracciones se obtuvieron mediante fraccionamiento líquido-líquido y la purificación de punicalagina se realizó en una columna cromatográfica llena de Diaion® HP-20. Se utilizaron HPLC y RMN (1H y 13C -Acetona-d6) para la identificación, cuantificación y elucidación estructural de punicalagina y se determinó MIC (CLSI-M7A10) para 23 aislamientos clínicos. El contenido de punicalagina fue mayor en el extracto y fracciones de la variedad granate, alcanzando el 81,5% en la muestra de punicalagina purificada, que mostró buena actividad antimicrobiana frente a aislados clínicos, con énfasis en *S. aureus* y *S. schleiferi schleiferi*, donde la CIM fue de 31,75. µg / ml. De esta forma. La punicalagina se definió como un metabolito importante para el potencial antimicrobiano de los frutos de *P. granatum*, sin embargo, el sinergismo de los metabolitos de las fracciones de acetato de etilo y la fracción acuosa de la variedad granate, tuvo una importancia considerable para la buena actividad antimicrobiana de estas fracciones, en comparación con 100 % del SCP y SCN. Así, los resultados obtenidos confirman la actividad antimicrobiana de los metabolitos presentes en la cáscara de la granada, lo que puede sustentar nuevas investigaciones sobre formulaciones farmacéuticas basadas en *P. granatum*, como alternativa para el tratamiento, prevención y control de la mastitis.

Palabras clave: Granada, Variedade granate; Variedade *wonderful*; Concentración Mínima Inhibitoria (CMI); Cromatografia Líquida de Alta Resolución (CLAR).

1. Introduction

Punica granatum L., belonging to the Lythraceae family and popularly known as pomegranate, originated in Asian countries such as Iran, Afghanistan, and India, and later started to be cultivated in the Mediterranean region, from where it broke European borders, reaching tropical regions such as South America (Moga et al., 2021; Melgarejo-Sanchez et al., 2021). It is usually found as an ornamental pomegranate, with white flowers, small fruits and hard seeds, or edible pomegranate, of medicinal value, with red flowers, large fruits and soft seeds (Ge et al., 2021).

Source of several biologically active compounds, pomegranate is rich in flavonoids, anthocyanins, ellagitannins, ellagic acid, gallic acid, punic acid, pedunculagin, punicalin, punicalagin, vitamins, terpenes, among others. Given its rich phytochemical profile, the studies highlight a range of pharmacological properties, such as antimicrobial, anti-inflammatory, antioxidant, antitumor, antidiabetic, antihypertensive and cardioprotective activities (Wang et al., 2018; Mahdavi et al., 2021; Shaikh & Bhandary, 2021).

The extract of medicinal plants is commonly appointed as an object of research for alternative purposes to the use of conventional antimicrobials (Shui et al., 2021). Thus, studies attribute the broad spectrum of antimicrobial activity of pomegranate, against gram-positive and gram-negative bacteria, to the abundance of polyphenols, such as flavonoids and tannins

(Ge et al., 2021). Among these compounds, punicalagin is the hydrolyzable tannin with the highest concentration in the bark of *P. granatum*, which can occur in α and β anomeric forms (Oudane et al., 2018; Venusova et al., 2021). Its antimicrobial potential is due to its ability to inactivate extracellular microbial proteins, through interaction with sulfhydryl groups, in addition to reducing the pH of the environment, due to the delocalization of electrons and consequent formation of protons, by hydroxyl groups present in its structure. In addition, punicalagin is capable of precipitating proteins from the protoplast and the plasma membrane, leading to a change in their functionality and consequent loss of cytoplasmic content, standing out as an important metabolite with antimicrobial activity (Kharchoufi et al., 2018; Bassari-Jahromi & Doostkam, 2019; Singh et al., 2019).

In this sense, one of the most important causes of impacts on animal health, milk production and quality is bovine mastitis. Defined as an inflammatory process in the mammary gland, mastitis is usually caused by bacterial agents such as *Staphylococcus* and *Streptococcus*. It can manifest in the form of Clinical Mastitis (CM), where signs of infection of the mammary gland are apparent, or Subclinical Mastitis (MS), diagnosed through tests based on somatic cell counts (Pedersen et al., 2021; Silva et al., al., 2021; Wang et al., 2021).

The consequences of bovine mastitis involve large economic losses for both producers and the dairy industry (Sharun et al., 2021). It is estimated that annually, around the world, around one million cows are compromised, generating losses in the order of 125 billion euros (Kabelitz et al., 2021). In addition to the expenses generated by the treatment, use of medication and employment of personnel, there is a concern with the quality of life of the animals, which can be slaughtered as a result of mastitis. Thus, measures to maintain the integrity of the mammary gland and animal welfare include hygiene practices, control of disease-causing pathogens, and attention to the animal's housing and milking conditions, to reduce the incidence of mastitis in the herd (Pedersen et al., 2021; Zigo et al., 2021).

The study of medicinal plants with an antimicrobial effect is an alternative for the treatment, prevention and control of diseases responsible for great social impact. Therefore, considering the pharmacological properties of pomegranate, this study aimed to evaluate the antimicrobial activity of the ethanol extract, fractions and purified punicalagin from the rind of the fruit of *P. granatum* (pomegranate), granada and wonderful varieties, against Coagulase-positive *Staphylococcus* (CPS) and Coagulase-negative *Staphylococcus* (CNS), isolated from cows with mastitis.

2. Methodology

2.1 Plant material

The fruits of *P. granatum* (Lythraceae family), from the granada and the wonderful varieties, were purchased at the Central de Abastecimento de Goiás (CEASA-GO), Goiânia-BR. After cleaning and removing the inside parts of the fruits, such as berries and seeds, the skins were dried in an oven with circulation and air renewal (Solab Científica® SL-102) at 40 °C for 3 days and then crushed in a Croton knife mill (Tecnal® TE-625). At the end of the process, the powder was stored at 25 °C, in a suitable container, protected from light.

2.2 *P. granatum* fruit peel extract

The powder of *P. granatum* fruits of the granada and wonderful varieties was macerated under constant agitation at 300rpm (Tecnal® TE 039-1), for 24 hours, in 80% ethanolic solvent. Under constant flow, 10 cycles of percolation were performed and, later, the ethanol extract was concentrated in a semi-industrial rotaevaporator (Buchi® R-220 SE). Finally, the extract of the two varieties was collected and stored at -22 °C, protected from light.

2.3 Liquid-Liquid fractionation and punicalagin purification

The fractionation of the extract of the granada and wonderful varieties was carried out in a separating funnel, using increasing polarity solvents, such as hexane, dichloromethane and ethyl acetate. After separating the fractions and evaporating the organic solvent, the Hexanic (Hex.F.), Dichloromethane (Dcm.F.), Ethyl Acetate (E.Ac.F.) and Aqueous (Aq.F.) fractions, were lyophilized and stored protected from light at 25 °C. Punicalagin was purified on a chromatographic column (28 x 4 cm), filled with Diaion® HP-20 vinyl polymer gel (Sigma, USA) and loaded with 62 g of Aq.F. of the granada variety. The mobile phase used was water (100%); water/methanol in the ratios of 8:2; 6:4; 4:6 and 2:8; and methanol (100%). At the end of elution, aliquots were collected, lyophilized and stored away from light.

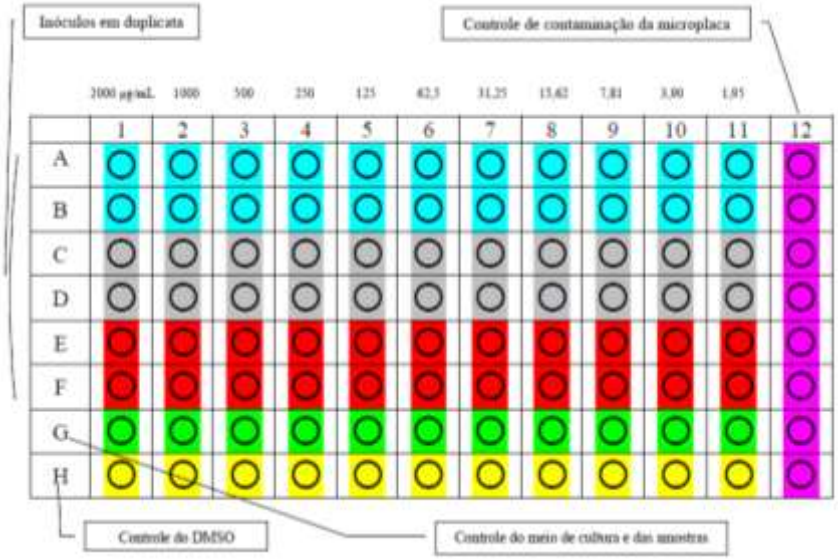
2.4 Identification, quantification and structural elucidation of punicalagin

HPLC was used to identify and quantify punicalagin in the extract and fractions of *P. granatum*, granada and wonderful varieties, as well as in the aliquots obtained by column chromatography. The chromatographic analysis was carried out in a Waters e2695 (Alience), equipped with a Diode Arrangement Detector (DAD) e2998 and an Empower 2.0 data processing system. The stationary phase was a Waters XTerra® C18 column (250 x 4.6 mm, 5 µm), injection volume 10 µL, operating temperature 40 °C, mobile phase flow 1.0 mL/min, detection at wavelength 260 nm and elution time of 25 min. The mobile phase was composed of acetonitrile (A) and 0.01 M (v/v) phosphate buffer (B), using the following elution gradient: 5% A and 95% B in 5 min; 15% A and 85% B in 10 min; 30% A and 70% B in 16 min; 3% A and 97% B in 18 min and 3% A and 97% B in 25 min as described by Chaibub et al. (2020), with adaptations. After purification by column chromatography, the structural elucidation of punicalagin was determined by 1D and 2D Nuclear Magnetic Resonance (NMR) in a Bruker Avance III 500 spectrometer, operating at a frequency of 500.13 MHz for ^{1}H and 125.76 MHz for the ^{13}C.

2.5 Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined for inocula supplied by the department of bacteriology of the Institute of Tropical Pathology and Public Health of the Federal University of Goiás (IPTSP/UFG). We used 14 CPS strains of the species *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Staphylococcus hyicus*, *Staphylococcus schleiferi schleiferi* and *Staphylococcus schleiferi coagulans*, and 9 CNS strains, totaling 23 clinical isolates from cows with mastitis. The study was performed based on the description of the Clinical and Laboratory Standard Institute (CLSI-M7A10). Thus, 20 mg of each sample was previously diluted in dimethylsulfoxide (DMSO) and serially distributed in 96-well microplates, filled with Müller Hinton broth, starting from a concentration of 2000 µg/mL to 1.95 µg/mL. line G was used as a control for contamination of the culture medium and samples, while line H was the DMSO control. Column 12 was used as a control for microplate contamination. Inoculums were distributed in duplicate on the microplates and incubated at 35ºC±2 for 24 hours (Figure 1). Finally, Triphenyl Tetrazolium Chloride (TTC) developer was added to each well of the plate and MIC was expressed as the first well free of red staining. Antimicrobial activity was classified as described by Alves et al. (2021), where it is considered good activity when MIC ≤ 125 µg/mL, moderate when MIC is between 126 and 500 µg/mL, weak when MIC is between 501 and 1000 µg/mL, and inactive for MIC >1000 µg /mL.

Figure 1. Schematic model for MIC determination in 96-well microplates. Inoculums were distributed in duplicate, line G was used as a control for contamination of the culture medium and samples, while line H was the control of DMSO and column 12 was used as a control for contamination of the microplate.



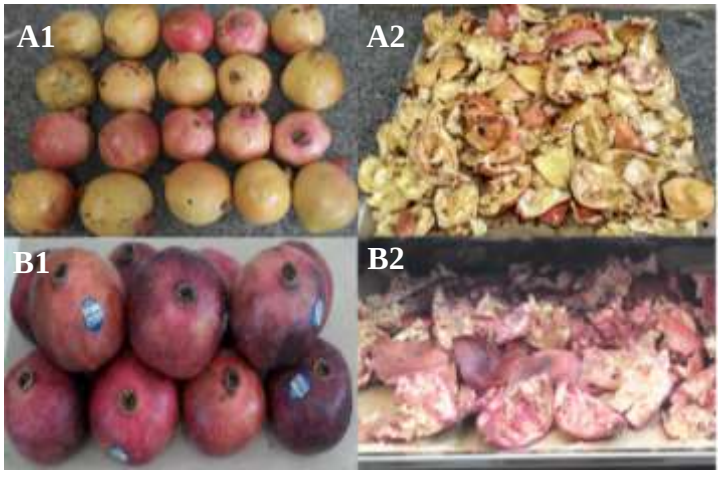
Source: Authors.

3. Results and Discussion

3.1 Plant material

The fruits of *P. granatum*, granada and wonderful varieties, had an oval shape, peculiar odor, smooth skin surface and crown-like upper structure, as described by The Ayurvedic Pharmacopoeia of India (API, 2004). The skin color varied between shades of yellow, for the granada variety, to red, for the wonderful variety, which was considerably larger than the fruits of the grana variety (Figure 2). At the end of the drying process, 30.46% of the plant material of the granada variety and 30.89% of the wonderful variety was recovered, which is in accordance with the literature, since after conditioning the fresh raw material for drying, the loss of humidity can reach 75% (Borgo et al., 2010).

Figure 2. General aspects of *P. granatum* fruits and peels, granada and wonderful varieties: A1-*P. granatum* fruits, granada variety; A2-*P. granatum* fruit peel, granada variety; B1-*P. granatum* fruits, wonderful variety; B2-*P. granatum* fruit peel, wonderful variety.



Source: Authors.

3.2 Extract from the peel of P. granatum fruits

The extraction solvent was determined based on the study by Loures (2013), which optimized the extraction of tannins present in the pomegranate peel, based on the use of ethanolic solvents in different proportions. According to Sridhar et al. (2021), due to its advantages, such as low cost, low amount of energy used and simplicity, maceration is one of the most used methods for the extraction of phenolic compounds. Furthermore, prior use of this technique helps to soften tissues and facilitate extraction. Therefore, the joint use of the percolation technique reinforces the diffusion and osmosis movement of the secondary metabolites present in the vegetable raw material, to the solvent (Naviglio et al., 2019). Thus, the choice of the appropriate extraction solvent for better tannin extraction, combined with low cost and simple extraction techniques, proved to be efficient in obtaining the ethanol extract of the peel of *P. granatum* fruits, of the granada and marvelous varieties.

3.3 Liquid-liquid fractionation and purification of punicalagin

Even when dealing with the same species, medicinal plants of different varieties can show variability in the proportion and concentration of secondary metabolites. Furthermore, the phytochemical profile can be influenced by factors such as seasonality, temperature, availability of water and nutrients, the incidence of ultraviolet radiation, altitude, among others (Gobbo-Neto & Lopes, 2007; Rezende et al., 2015). Thus, it was noted that the yield of Hex.F. and Dcm.F. of the granada variety were higher than that observed for the wonderful variety fractions, while the E.Ac.F. and Aq.F. showed similar yields in both varieties (Table 1).

For the purification of punicalagin, a chromatographic column packed with Diaion® HP-20 was chosen, due to its hydrophobic character and the large contact surface, ideal for purification of high molecular weight organic compounds (Sa et al., 2018; Wang et al., 2019; Santos et al., 2021). The Aq.F. of the granada variety was chosen for purification, as it confers good yield and good antimicrobial capacity in previously performed MIC tests. These factors suggest that the phytochemical profile of the granada variety of pomegranate presents a relevant concentration of bioactive compounds responsible for the antimicrobial activity, which would explain the good performance observed for F.Aq. of this variety. At the end of the chromatography, 141 aliquots (20 mL) were collected and named A.1 to A.141.

Table 1. Yield (%) of Hex.F., Dcm.F., E.Ac.F. and Aq.F., after liquid-liquid partition of the peel extract of *P. granatum*, granada and wonderful varieties.

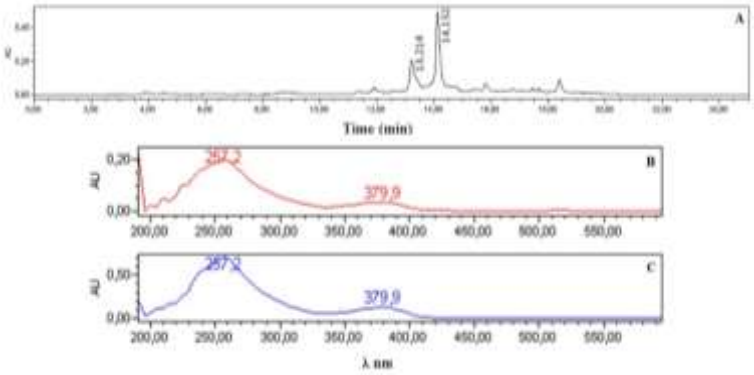
	***P. granatum* granada variety**	***P granatum wonderful* variety**
Hex.F.	3.7 %	1.46 %
Dcm.F.	5.78 %	0.38 %
E.Ac.F.	14.88 %	15.38 %
Aq.F.	77.49 %	82.78 %

Subtitle: Hex.F.: Hexanic Fraction; Dcm.F.: Dichloromethane Fraction; E.Ac.F.: Ethyl Acetate Fraction; Aq.F.: Aqueous Fraction. Source: Authors.

3.4 Identification, quantification and structural elucidation of punicalagin

The chromatographic method allowed the identification and quantification of punicalagin in *P. granatum* samples. Typically, the α and β anomeric structures of the hydrolyzable tannins show up as two peaks in the chromatograms (Chaibub et al., 2020). Thus, the chromatographic profile of the *P. granatum* extract showed two intense peaks with a retention time of 13.214 min and 14.132 min, respectively (Figure 3A). The maximum absorption bands were 257.2 and 379.9 nm, identical for the two peaks (Figure 3B and 3C), suggesting that the peaks refer to the α and β anomers of punicalagin, according to the punicalagin absorption spectrum reported in the literature, such as 258 and 380 nm (Hernandez-Corroto et al., 2019); 260 and 380 nm (Gosset-Erard et al., 2021); and 254 and 378 nm (Veloso et al., 2020).

Figure 3. A. Chromatographic profile of *P. granatum* extract, highlighting the retention time of peak 1 at 13.214 min and peak 2 at 14.132 min; B. Absorption spectrum at 260 nm of peak 1 of *P. granatum* extract; C. Absorption spectrum at 260 nm of peak 2 of *P. granatum* extract.



Source: Authors.

The punicalagin content in the extract and fractions of *P. granatum*, granada and wonderful varieties, as well as in the aliquot purified by column chromatography, is shown in Table 2. The punicalagin content in the extract of the granada variety was 20.8%, while for the wonderful variety it was only 8.5%. The study of different varieties of plants of the same species may indicate variation in their phytochemical composition and the consequent possibility of variation in therapeutic, nutraceutical and attractiveness effects, such as smell, color and size characteristics (Melgarejo-Sanchez et al., 2021). In this sense, the quantitative and qualitative monitoring of five pomegranate cultivars, carried out by Di Stefano et al. (2019), pointed out statistically significant differences between its phenolic compounds. Furthermore, the study of the nutritional characteristics of the pomegranate as a function of the maturation stage of seven cultivars showed an accentuated concentration of punicalagin in acidic and semi-acidic varieties, to the detriment of sweet ones (Tozzi et al., 2021).

The liquid-liquid fractionation aimed at the partial purification of secondary metabolites, through the affinity between the analyte and extracting solvent. Therefore, bioactive compounds with greater polarity, such as tannins and flavonoids, were mostly expected in E.Ac.F. and Aq.F. (Cechinel Filho & Yunes, 1998; Fernandes et al., 2019). Thus, it was observed that the punicalagin content in Hex.F. was similar in both varieties, while in Dcm.F., the metabolite was identified only in the granada variety. For fractions E.Ac.F. and Aq.F., the punicalagin content was higher in the granada variety, with emphasis on the Aq.F., which exceeded by more than 2 times the content observed for the Aq.F. of the wonderful variety.

Table 2. Punicalagin content in the extract and fractions of *P. granatum*, granada and wonderful varieties, as well as for the purified aliquot of punicalagin.

	P. granatum granada variety (Content %)	*P. granatum* wonderful variety (Content %)
Extract	20.8	8.5
Hex.F.	2.5	2.3
Dcm.F.	4.7	-
E.Ac.F.	18.6	14.3
Aq.F.	17.1	7
Purified Punicalagin	81.5	-

Subtitle: Hex.F.: Hexanic Fraction; Dcm.F.: Dichloromethane Fraction; E.Ac.F.: Ethyl Acetate Fraction; Aq.F.: Aqueous Fraction.
Source: Authors.

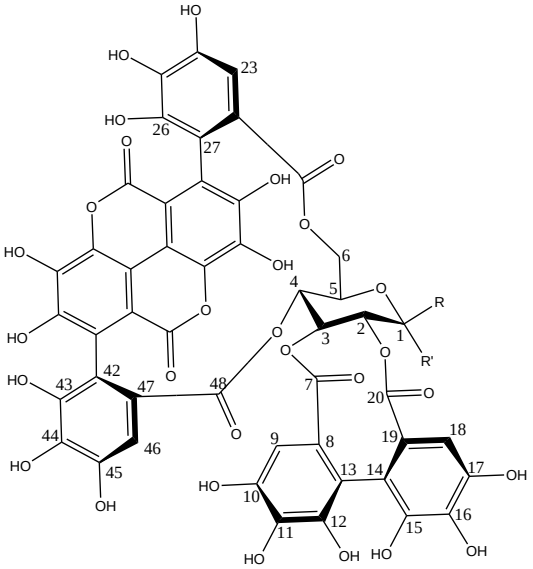
The identification of the chromatographic profiles of the purified aliquots pointed to the presence of α and β anomers of punicalagin in aliquots A.73-85, referring to the use of the mobile phase H_2O/Methanol (8:2). After quantification, the content

of 81.5% punicalagin in the A.78 aliquot was reached, confirming the efficiency of column chromatography for purification. A similar result was obtained in aqueous two-phase systems, however, the high cost of some polymers, associated with slow phase separation, are some of the factors that limit the application of this technique (Indurkar & Rathod, 2018; Chen et al., 2020). Furthermore, systems sized to achieve purity greater than 99% are expensive, as is the case with the preparative HPLC technique (Oudane et al., 2018; Sun et al., 2021).

The ^{1}H NMR spectrum of purified punicalagin (A-78) contains a doublet in 5.1 (*J* 3.49) coupled with ^{13}C 89.2, corresponding to the resonance set of the α-anomer of punicalagin, and a doublet in 4.78 (*J* 8.03) coupled with ^{13}C 93.5 of the glucose ring, corresponding to the β anomer of punicalagin. The 3.49 Hz coupling constant of the glucose anomeric resonance (H1) at 5.1 ppm allows the assignment of the most intense resonances to the α anomer of punicalagin. The attribution to glucose protons (δ ^{1}H 2 to 5) was evidenced in the form of multiplets and triplets in the region between 2.69 and 5.22. For the ^{13}C resonance assignments of the sugar moiety, the spectrum provided the bond correlations pointed out by HMBC (Heteronuclear Multiple Bond Correlation) and HSQC (Heteronuclear Single Quantum Coherence). Signals of punicalagin aromatic protons were performed by observing the correlation by HMBC and observed between carbonyl carbons and aromatic and aliphatic protons. Interactions between gallagic acid H23 and ellagic acid H18 were observed with the glucose of each anomer. The attributions of ^{1}H and ^{13}C resonance (Acetone-d6) and structure of α- and β-punicalagin are shown in table 3, and the results were compared to those in the literature (Kraszni et al., 2013; Carneiro et al., 2016).

Table 3. ^{1}H and ^{13}C NMR(Acetone-d6) assignments and structure with enumeration scheme of α- and β-punicalagin.

	δ ^{1}H (*J* in Hz)		δ^{13}C	
Position	Anomer α	Anomer β	Anomer α	Anomer β
1	5.1 (*d*, 3.49)	4.78 (*d*, 8.03)	89.2	93.5
2	4.7 a 4.8 (*m*)	5.22 (*t*, 9.45)	78.0	76.2
3	4.89 (*t*, 9.32)	4.89 (*t*, 9.32)	74.0	78.5
4	4.65 (*s*)	4.8 (*m*)	75.9	73.6
5	3.27 (*d*, 1.44)	2.69 (*td*, 1.76, 9.87)	66.0	71.7
6a	4.15 (*q*, 10.62)	4.15 (*q*, 10.62)	63.4	63.4
6b	2.11 (*m*)	2.2 (*m*)	63.4	63.4
7			168.0	168.0
8			123.5	123.5
9	6.518 (s)	6.517 (s)	104.4	106.5
10			144.5	144.5
11			135.2	134.8
12			144.5	144.5
13			113.8	112.9
14			113.8	113.8
15			144.5	144.5
16			135.2	135.2
17			144.5	144.5
18	6.586 (s)	6.588 (s)	106.6	106.8
19			123.5	123.5
20			168.0	168.0
23	6.99 (s)	7.00 (s)	109.1	109.5
26			138.4	138.4
27			111.4	111.4
42			113.8	117.0
43			144.5	144.7
44			135.4	136.8
45			144.5	144.7
46	6.60 (s)	6.66 (s)	108.8	110.9
47			123.5	123.5
48			168.0	167.0



Source: Authors.

3.5 Determination of Minimum Inhibitory Concentration (MIC)

MIC can be defined as the lowest concentration necessary for an antimicrobial agent to be able to inhibit the growth of a microorganism (Tan et al., 2021). In our study, the use of the broth microdilution technique was of great importance, as it is the most sensitive method for promoting quantitative results on antimicrobial activity. Other techniques, such as diffusion in a petri dish, only show qualitative results, limiting themselves to preliminary data (Nascimento et al., 2007; Scorzoni et al., 2016).

The MIC of the extract and fractions of *P. granatum*, granada and wonderful varieties, as well as aliquot A. 78, are shown in Table 4. The samples of ethanol extract of the two studied pomegranate varieties showed antimicrobial potential, with a MIC of 125 μg/mL for at least one strain of the species tested, except for the two subspecies of *S. schleiferi*. Thus, the antimicrobial capacity of the ethanol extract of the granada variety was predominantly moderate, while for the wonderful variety it was moderate to good, for 39.1% and 43.5% of the strains tested (Table 5). Studies with the pomegranate peel extract attributed MIC of 171 μg/mL against *S. aureus* and 555 μg/mL against *Salmonella enterica* (Fourati et al., 2019), 62.500 μg/mL for *Salmonella typhimurium* (Tadi et al., 2020), 10000 μg/ml for *Escherichia coli* and *Clostridium perfringens* (Skenderidis et al., 2019) and 1870 μg/ml against *Listeria monocytogenes* (Demir, 2021). Variations observed in the inhibition profiles of the *P. granatum* bark extract can be attributed to plant variety, choice of extraction solvent, geographic position of the planting and consequent exposure to different climatic conditions (Choi et al., 2011). Thus, the results obtained for the extract samples confirm the pomegranate's ability to delay the development and growth of pathogenic microorganisms, in addition to an eventual impairment of the morphology and integrity of the bacterial membrane (Sateriale et al., 2020).

Polyphenols and tannins, the main metabolites responsible for the antimicrobial activity of pomegranate (Ko et al., 2021), tend to have low concentration in Hex.F and Dcm.F, since they are non-polar character fractions. Thus, no antimicrobial potential of these fractions was observed against the tested microorganisms. The E.Ac.F. and Aq.F. of the granada variety, reached MIC values ranging from 31.25 μg/mL to 125 μg/mL, showing good activity against 100% of the strains tested. However, like the granada variety, the same efficiency was not verified for the wonderful variety fractions, where E.Ac.F. and Aq.F. even presented weak or inactive MIC against strains of the species *S. aureus*, *S. hyicus*, *S. schleiferi coagulans* and CNS (Tables 4 and 5).

If compared with E.Ac.F. and Aq.F. of the granada variety, purified punicalagin was more effective against strains of *S. aureus* and *S. schleiferi schleiferi*, reaching a MIC of up to 31.75 μg/mL. For the other species, the punicalagin MIC was the same observed for at least a fraction of the granada variety, except for the CNS, *3*, *4* and *6* strains (Table 4). Thus, punicalagin is confirmed as a metabolite of great importance for expression of the antimicrobial potential of pomegranates (Rongai et al., 2019; Gosset-Erard et al., 2021). The E.Ac.F. and Aq.F. of the granada variety, received the same antimicrobial classification as the purified punicalagin, showing good activity against 100% of the evaluated microorganisms (Table 5). Such occurrence may be related to the synergistic effect of secondary metabolites, noticed in conditions where only semi-purification of phyto complex compounds occurs, as it happens in E.Ac.F. and Aq.F. (Fawole et al., 2012; Casanova & Costa, 2017).

Table 4. MIC of extract and fractions of *P. granatum*, granada and wonderful varieties, as well as purified punicalagin, against CPS and CNS, isolated from cows with mastitis.

	MIC Extract (µg/mL)		**MIC Fractions (µg/mL)**				**MIC purified punicalagin (µg/mL)**
			Granada		*Wonderful*		
STRAINS	Granada	*Wonderful*	E.Ac.F.	Aq.F.	E.Ac.F.	Aq.F.	A.78
S. aureus							
(SA1)	250	250	62.5	62.5	125	500	31.25
(SA2)	500	125	62.5	62.5	125	500	62.5
(SA3)	250	250	62.5	62.5	125	2000	31.25
(SA4)	125	250	62.5	62.5	125	500	31.25
(SA5)	2000	125	62.5	62.5	500	2000	62.5
(SA6)	500	125	62.5	62.5	125	2000	31.25
S. haemolyticus							
(SH1)	250	125	62.5	125	125	125	62.5
(SH2)	500	125	62.5	62.5	125	125	62.5
S. hyicus							
(SHY1)	1000	125	125	62.5	1000	500	62.5
(SHY2)	500	250	62.5	62.5	500	2000	62.5
(SHY3)	250	125	62.5	62.5	125	500	62.5
S. schleiferi_schleiferi							
(SSS1)	1000	250	125	62.5	1000	1000	62.5
S. schleiferi coagulans							
(SSC1)	500	2000	62.5	125	1000	1000	125
(SSC2)	500	250	62.5	62.5	2000	2000	62.5
SCN							
(SCN1)	1000	250	125	62.5	1000	2000	62.5
(SCN2)	500	250	62.5	62.5	250	250	62.5
(SCN3)	500	1000	62.5	62.5	1000	1000	125
(SCN 4)	500	250	62.5	62.5	2000	2000	125
(SCN 5)	125	125	31.25	31.25	125	125	31.25
(SCN 6)	250	125	62.5	31.25	250	250	62.5
(SCN 7)	500	125	31.25	62.5	125	250	31.25
(SCN 8)	250	1000	62.5	62.5	500	1000	125
(SCN 9)	500	1000	62.5	62.5	500	500	62.5

Subtitle: E.Ac.F.: Ethyl acetate fraction; Aq.F.: Aqueous fraction; A.78: Purified Punicalagin. Source: Authors.

Table 5. Antimicrobial capacity of extract and fractions of *P. granatum*, granada and wonderful varieties, as well as purified punicalagin, against CPS and CNS, isolated from cows with mastitis.

Intensity	**Extract**		**Fractions**				**Purified punicalagin**
	Granada	Wonderful	Granada		Wonderful		A.78
			E.Ac.F.	Aq.F.	E.Ac.F.	Aq.F.	
Good	8.7%	43.5%	100%	100%	43.5%	13%	100%
Moderate	73.9%	39.1%	-	-	26.1%	39.1%	-
Weak	13%	13%	-	-	21.7%	17.4%	-
Inactive	4.3%	4.3%	-	-	8.7%	30.4%	-

Source: Authors.

4. Conclusion

The appearance of the fruits of *P. granatum* was satisfactory for the development of this study. After drying, the use of maceration and percolation methods, combined with the appropriate solvent, proved to be efficient for extracting tannins from the vegetable raw material, in addition to attributing simplicity and low cost to the technique. The liquid-liquid fractionation enabled the semi-purification of secondary metabolites and obtaining organic fractions of the ethanol extract of pomegranate, of the granada and wonderful varieties. Purification of Aq.F. of the granada variety was efficient, reaching 81.5% of purified punicalagin. With a MIC of 31.75 µg/mL, purified punicalagin was effective against clinical isolates, especially *S. aureus* and *S. schleiferi schleiferi*. Thus, punicalagin was determined as a metabolite of great importance for the expression of the antimicrobial

potential of *P. granatum* fruits. However, the synergism between the secondary metabolites of the E.Ac.F. and Aq.F. fractions of the granada variety was observed, since, under semi-purification conditions of the extract, the antimicrobial activity was good for 100% of the CPS and CNS, just like for punicalagin. Thus, the potential of *P. granatum* fruits as a source of secondary metabolites with antimicrobial potential against clinical isolates from cows with mastitis was confirmed. Thus, the results obtained in this study can support research on pharmaceutical formulations based on *P. granatum*, as an alternative for the treatment, prevention and control of mastitis.

Acknowledgments

The authors are grateful to the anonymous reviewer for the comments, which helped us to improve the manuscript. This study received financial support from the Coordination for the Improvement of Higher Education Personnel (CAPES) and the National Council for Scientific and Technological Development (CNPq), the Foundation for Research Support of the State of Goiás (FAPEG) and National Institute of Science and Technology of Host Pathogen Interaction. This study was financed in part by the CAPES, Finance Code 001.

References

Alves, D. N., Ferreira, A. R., Duarte, A. B. S., Melo, A. K. V., Sousa, D. P. & Castro, R. D. (2021). Breakpoints for the Classification of Anti-Candida Compounds in Antifungal Screening. *BioMed Research International*, 2021. https://doi.org/10.1155/2021/6653311.

API. (2004). *The Ayurvedic Pharmacopoeia of India*. (Part I, Volume IV). India. Department of Indian Systems of Medicine and Homoeopathy, Ministry of Health and Family Welfare. http://www.ayurveda.hu/api/API-Vol-4.pdf.

Bassari-Jahromi, S. & Doostkam, A. (2019). Comparative evaluation of bioactive compounds of various cultivars of pomegranate (Punica granatum) in different world regions. *Agriculture and Food*, 4(1), 41-55. https://doi.org 10.3934/agrfood.2019.1.41.

Borgo, J., Xavier, C. A. G., Moura, D. J., Richter, M. F. & Suyenaga, E. S. (2010). Influência dos processos de secagem sobre o teor de flavonoides e na atividade antioxidante dos extratos de *Baccharis articulata* (Lam.) Pers., Asteraceae. *Brazilian Journal of Pharmacognosy*, 20(1), 12-17. https://doi.org/10.1590/S0102-695X2010000100004.

Carneiro, C. C., Santos, S. C., de Souza, L. R. Jr., Bara, M. T. F., Chaibub, B. A., de Melo Reis, P. R., Chaves, D. A., da Silva, A. J. R., Silva, L. S., de Melo e Silva, D. & Chen-Chen, L. (2016). Chemopreventive effect and angiogenic activity of punicalagin isolated from leaves of *Lafoensia pacari* A. St.-Hil. *Toxicology and Applied Pharmacology*, 310, 1-8. 10.1016/j.taap.2016.08.015.

Casanova, L. M. & Costa, S. S. (2017). Interações sinérgicas em produtos naturais: potencial terapêutico e desafios. *Revista Virtual de Química*, 9(2), 575-595. Retrieved from http://static.sites.sbq.org.br/rvq.sbq.org.br/pdf/v9n2a09.pdf.

Cechinel Filho, V. & Yunes, R. A. (1998). Estratégias para a obtenção de compostos farmacologicamente ativos a partir de plantas medicinais. conceitos sobre modificação estrutural para otimização da atividade. *Química Nova*, 21(1). https://doi.org/10.1590/S0100-40421998000100015.

Chaibub, B. A., Parente, L. M. L., Lino Jr, R. S., Cirilo, H. N. C., Garcia, S. A. S., Nogueira, J. C. M., Conceição, E. C., Thomaz, D. V., Santos, S. C. & Bara, M. T. F. (2020). Pharmacognosy Investigation of wound healing activity of Lafoensia pacari (Lythraceae) leaves extract cultivated in Goiás state, Brazil. *Rodriguesia*, 71. https://doi.org/10.1590/2175-7860202071058.

Chen, J., Liao, C., Ouyang, X., Kahramanoglu, I., Gan, Y. & Li, M. (2020). Antimicrobial Activity of Pomegranate Peel and Its Applications on Food Preservation. *Journal of Food Quality*, 2020. https://doi.org/10.1155/2020/8850339.

Choi, J. G., Kang, O. H., Lee, Y. S., Chae, H. S., Oh, Y. C., Brice, O. O., Kim, M. S., Sohn, D. H., Kim, H. S., Park, H., Shin, D. W., Rho, J. R. & Kwon, D. Y. (2011). In Vitro and In Vivo Antibacterial Activity of Punica granatum Peel Ethanol Extract against Salmonella. *Evidence-Based Complementary and Alternative Medicine*, 2011. 10.1093/ecam/nep105.

CLSI. (2018). *Methodos for Dilution Antimicrobial Susceptibility Testes for Bacteria That Grow Aerobically*. (11th ed.) CLSI standard M07. Wayne, PA: Clinical and Laboratory Standard Institute.

Demir, T. (2021). Effects of Green Tea Powder, Pomegranate Peel Powder, Epicatechin and Punicalagin Additives on Antimicrobial, Antioxidant Potential and Quality Properties of Raw Meatballs. *Molecules*, 26(13), 4052. https://doi.org/10.3390/molecules26134052.

Di Stefano, V., Pitonzo, R., Novara, M. E., Bongiorno, D., Indelicato, S., Gentile, C., Avellone, G., Bognanni, R., Scandurra, S. & Melilli, M. G. (2019). Antioxidant activity and phenolic composition in pomegranate (*Punica granatum* L.) genotypes from south Italy by UHPLC–Orbitrap-MS approach. *Journal of the Sciente of Food and Agriculture*, 99(3), 1038-1045. https://doi.org/10.1002/jsfa.9270.

Fawole, O. A., Makunga, N. P. & Opara, U. L. (2012). Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. *BMC Complementary & Alternative Medicine*, 12 (1), 200. 10.1186/1472-6882-12-200.

Fernandes, D. A., Barros, R. P. C., Teles, Y. C. F., Oliveira, L. H. G., Lima, J. B., Scotti, M. T., Nunes, F. C., Conceição, A. S. & Souza, M. F. V. (2019). Larvicidal Compounds Extracted from *Helicteres velutina* K. Schum (Sterculiaceae) Evaluated against *Aedes aegypti* L. *Molecules*, 24(12), 2315. https://doi.org/10.3390/molecules24122315.

Fourati, M., Smaoui, S., Ennouri, K., Hlima, H. B., Elhadef, K., Chakchouk-Mtibaa, A., Sellem, I. & Mellouli, L. (2019). Multiresponse Optimization of Pomegranate Peel Extraction by Statistical versus Artificial Intelligence: Predictive Approach for Foodborne Bacterial Pathogen Inactivation. *Evidence-Based Complementary and Alternative Medicine*, 2019. https://doi.org/10.1155/2019/1542615.

Ge, S., Duo, L., Wang, J., Zhula, G., Yang, J., Li, Z. & Tu, Y. (2021). A unique understanding of traditional medicine of pomegranate, *Punica granatum* L. and its current research status. *Journal of Ethnopharmacology*, 271. https://doi.org/10.1016/j.jep.2021.113877.

Gobbo-Neto, L. & Lopes, N. P. (2007). Plantas medicinais: fatores de influência no conteúdo de metabólitos secundários. *Quimica Nova*, 30(2), 374-381. https://doi.org/10.1590/S0100-40422007000200026.

Gosset-Erard, C., Zhao, M., Lordel-Madeleine, S. & Ennahar, S. (2021). Identification of punicalagin as the bioactive compound behind the antimicrobial activity of pomegranate (*Punica granatum* L.) peels. *Food Chemistry*, 352. https://doi.org/10.1016/j.foodchem.2021.129396.

Hernandez-Corroto, E., Marina, M. L. & Garcia, M. C. (2019). Extraction and identification by high resolution mass spectrometry ofbioactive substances in different extracts obtained frompomegranate peel. *Journal of Chromatography A*, 1594, 82-92. https://doi.org/10.1016/j.chroma.2019.02.018.

Indurkar, S. J. & Rathod, V. K. (2018). Aqueous two-phase extraction of punicalagin (α+β) from pomegranate peel by response surface methodology. *Separation Science and Technology*, 54(1), 51-58. https://doi.org/10.1080/01496395.2018.1488866.

Kabelitz, T., Aubry, E., Vorst, K. V., Amon, T. & Fulde, M. (2021). The Role of Streptococcus spp. in Bovine Mastitis. *Microorganism*, 9(7), 1497. https://doi.org/10.3390/microorganisms9071497.

Kharchoufi, S., Licciardello, F., Siracusa, L., Muratore, G., Hamdi, M. & Restuccia, C. (2018). Antimicrobial and antioxidant features of 'Gabsi' pomegranate peel extracts. *Industrial Crops & Products*, 111, 345-352. https://doi.org/10.1016/j.indcrop.2017.10.037.

Ko, K., Dadmohammadi, Y. & Abbaspourrad, A. (2021). Nutritional and Bioactive Components of Pomegranate Waste Used in Food and Cosmetic Applications: A Review. *Foods*, 10(3), 657. https://doi.org/10.3390/foods10030657.

Kraszni, M., Marosi, A. & Larive, C. K. (2013). NMR assignments and the acid–base characterization of the pomegranate ellagitannin punicalagin in the acidic pH-range. *Analytical and Bioanalytical Chemistry*, 405(17), 5807-5816. https://doi.org/10.1007/s00216-013-6987-x.

Loures, P. K. R. (2013). Desenvolvimento tecnológico de extratos vegetais padronizados a partir das cascas dos frutos de *Punica granatum* L. (lythraceae). (Dissertação de Mestrado, Programa de Pós-graduação em Ciências Farmacêuticas) Universidade Federal de Goiás, Faculdade de Farmácia, Goiânia, Goiás.

Mahdavi, A. M., Seyedsadjadi, N. & Javadivala, Z. (2021). Potential effects of pomegranate (Punica granatum) on rheumatoid arthritis: A systematic review. *The International Journal of Clinical Practice*, 75(8). https://doi.org/10.1111/ijcp.13999.

Melgarejo-Sanchez, P., Nunez-Gomez, D., Martinez-Nicolas, J. J., Hernandez, F., Legua, P. & Melgarejo, P. (2021). Pomegranate variety and pomegranate plant part, relevance from bioactive point of view: a review. *Bioresources and Bioprocessing*, 8(2). https://doi.org/10.1186/s40643-020-00351-5.

Moga, M. A., Dimienescu, O. G., Balan, A., Dima, L., Toma, S. I., Bigiu, N. F. & Blidaru, A. (2021). Pharmacological and Therapeutic Properties of Punica granatum Phytochemicals: Possible Roles in Breast Cancer. *Molecules*, 26(4). https://doi.org/10.3390/molecules26041054.

Nascimento, P. F. C., Nascimento, A. C., Rodrigues, C. S., Antoniolli, A. R., Santos, P. O., Barbosa Junior, A. M. & Trindade, R. C. (2007). Atividade antimicrobiana dos óleos essenciais: uma abordagem multifatorial dos métodos. *Brazilian Journal of Pharmacognosy*, 17(1), 108-113. https://doi.org/10.1590/S0102-695X2007000100020.

Naviglio, D., Scarano, P., Ciaravolo, M. & Gallo, M. (2019). Rapid Solid-Liquid Dynamic Extraction (RSLDE): A Powerful and Greener Alternative to the Latest Solid-Liquid Extraction Techniques. *Foods*, 8(7), 245. 10.3390/foods8070245.

Oudane, B., Boudemagh, D., Bounekhel, M., Sobhi, W., Vidal, M. & Broussy, S. (2018). Isolation, characterization, antioxidant activity, and proteinprecipitating capacity of the hydrolyzable tannin punicalagin from pomegranate yellow peel (*Punica granatum*). *Journal of Molecular Structure*, 1156, 390-396. https://doi.org/10.1016/j.molstruc.2017.11.129.

Pedersen, R. R., Kromker, V., Bjarnsholt, T., Pedersen, K. D., Buhl, R. & Jorgensen, E. (2021). Biofilm Research in Bovine Mastitis. *Frontiers in Pharmacology*, 8, 656810. 10.3389/fvets.2021.656810.

Rezende, W. P., Borges, L. L., Santos, D. L., Alves, N. M. & Paula, J. R. (2015). Effect of Environmental Factors on Phenolic Compounds in Leaves of Syzygium jambos (L.) Alston (Myrtaceae). *Modern Chemistry & Applications*, 3(2). 10.4172/2329-6798.1000157.

Rongai, D., Pulcini, P., Di Lernia, G., Nota, P., Preka, P. & Milano, F. (2019). Punicalagin Content and Antifungal Activity of Different Pomegranate (*Punica ganatum* L.) Genotypes. *Horticulturae*, 5(52). https://doi.org/10.3390/horticulturae5030052.

Sa, S., Chaul, L. T., Alves, V. F., Fiuza, T. S., Tresvenzol, L. M. F., Vaz, B. G., Ferri, P. H., Borges, L. L. & Paul, J. R. (2018). Phytochemistry and antimicrobial activity of Campomanesia adamantium. *Brazilian Journal of Pharmacognosy*, 28(3), 303-311. https://doi.org/10.1016/j.bjp.2018.02.008.

Santos, A. C. M., oliveira, V. C., Macedo, A. P., Bastos, J. K., Ogasawara, M. S., Watanabe, E., Chaguri, I. M., Silva-Lovato, C. H. & Paranhos, H. F. O. (2019). Effectiveness of Oil-Based Denture Dentifrices-Organoleptic Characteristics, Physicochemical Properties and Antimicrobial Action. *Antibiotics*, 10(7), 813. https://doi.org/10.3390/antibiotics10070813.

Sateriale, D., Facchiano, S., Colicchio, R., Pagliuca, C., Varricchio, E., Paolucci, M., Volpe, M. G., Salvatore, P. & Pagliarulo, C. (2020). In vitro Synergy of Polyphenolic Extracts From Honey, Myrtle and Pomegranate Against Oral Pathogens, S. mutans and R. dentocariosa. *Frontiers in Microbiology*, 11. https://doi.org/10.3389/fmicb.2020.01465.

Scorzoni, L., Sangalli-Leite, F., Singulani, J. L., Silva, A. C. A. P., Costa-Orlandi, C. B., Fusco-Almeida, A. M. & Mendes-Giannini, M. J. M. (2016). Searching new antifungals: The use of in vitro and in vivo methods for evaluation of natural compounds. *Journal of Microbiological Methods*, 123, 68-78. https://doi.org/10.1016/j.mimet.2016.02.005.

Shaikh, S. B. & Bhandary, Y. P. (2021). Therapeutic properties of Punica granatum L (pomegranate) and its applications in lung-based diseases: A detailed review. *Jounal of Food Biochemistry*, 45(4). https://doi.org/10.1111/jfbc.13684.

Sharun, K., Dhama, K., Tiwari, R., Gugjoo, M. B., Yatoo, M. I., Patel, S. K., Pathak, M., Karthik, K., Khurana, S. K., Singh, R., Puvvala, B., Amarpal., Singh, R., Singh, K. P. & Chaicumpa, W. (2021). Advances in therapeutic and managemental approaches of bovine mastitis: a comprehensive review. *Veterinary Quarterly*, 41(1), 107-136. https://doi.org/10.1080/01652176.2021.1882713.

Shui, Y., Li, J., Lyu, X. & Wang, Y. (2021). Phytotherapy in the management of denture stomatitis: A systematic review and meta-analysis of randomized controlled trials. *Phytotherapy Research*, 35(8). https://doi.org/10.1002/ptr.7073.

Silva, A. C., Laven, R. & Benite, N. R. (2021). Risk Factors AssociatedWith Mastitis in Smallholder Dairy Farms in Southeast Brazil. *Animals*, 11(7), 2089. https://doi.org/10.3390/ani11072089.

Singh, B., Singh, J. P., Kaur, A. & Singh, N. (2019). Antimicrobial potential of pomegranate peel: a review. *International Journal of Food Science and Technology*, 54(4), 959-965. https://doi.org/10.1111/ijfs.13964.

Sridhar, A., Ponnuchamy, M., Kumar, P. S., Kapoor, A., Vo, D. V. N. & Prabhakar, S. (2021). Techniques and modeling of polyphenol extraction from food: a review. *Environmental Chemistry Letters*, 19, 3409-3443. https://doi.org/10.1007/s10311-021-01217-8.

Skenderidis, P., Mitsagga, C., Giavasis, I., Petrotos, K., Lampakis, D., Leontopoulos, S., Hadjichristodoulou, C. & Tsakalof, A. (2019). The in vitro antimicrobial activity assessment of ultrasound assisted *Lycium barbarum* fruit extracts and pomegranate fruit peels. *Journal of Food Measurement and Characterization volume*, 13, 2017-2031. https://doi.org/10.1007/s11694-019-00123-6.

Sun, G., Abuduaini, M., Adili, G., Zhao, Y. & Aisa, H. A. (2021). Dual-tautomerism separation method based on asymmetric transformation: Gram-scale preparation of high-purity punicalagin from pomegranate peel wastes. *Journal of Chromatography A*, 1651. https://doi.org/10.1016/j.chroma.2021.462281.

Tadi, M., Boroujeni, H. M., Rafieian-Kopaei, M. & Sadrabad, E. K. (2020). Inhibitory effects of ethanolic extract of two Iranian pomegranates peel cultivars on Staphylococcus aureus and Salmonella typhimurium. *Asian Journal of Agriculture and Biology*, 8(3), 341-347.

Tan, R., Yu, A., Liu, Z., Liu, Z., Jiang, R., Wang, X., Liu, J., Gao, J. & Wang, X. (2021). Prediction of Minimal Inhibitory Concentration of Meropenem Against Klebsiella pneumoniae Using Metagenomic Data. *Frontiers in Microbiology*, 12. 10.3389/fmicb.2021.712886.

Tozzi, F., Nunez-Gomez, D., Legua, P., Del Bubba, M., Giordani, E. & Melgarejo, P. (2022). Qualitative and varietal characterization of pomegranate peel: High-value co-product or waste of production? *Scientia Horticulturae*, 291. https://doi.org/10.1016/j.scienta.2021.110601.

Veloso, F. S., Caleja, C., Calhelha, R. C., Pires, T. C. S., Alves, M. J., Barros, L., Genena, A. k., Barreira, J. C. M. & Ferreira, I. C. E. R. (2020). Characterization and Application of Pomegranate Epicarp Extracts as Functional Ingredients in a Typical Brazilian Pastry Product. *Molecules*, 25(7), 1481. https://doi.org/10.3390/molecules25071481.

Venusova, E., Kolesarova, A., Horky, P. & Slama, P. (2021). Physiological and Immune Functions of Punicalagin. *Nutrients*, 13(7), 2150. https://doi.org/10.3390/nu13072150.

Wang, D., Ozen, C., Abu-Reidah, I. M., Chigurupati, S., Patra, J. K., Horbanczuk, J. O., Józwik, A., Tzvetkov, N. T., Uhrin, P. & Atanasov, A. G. (2018). Vasculoprotective Effects of Pomegranate (*Punica granatum* L.). *Frontiers in Pharmacology*, 9(544). https://doi.org/10.3389/fphar.2018.00544.

Wang, S. T., Feng, Y. J., Lai, Y. J. & Su, N. w. (2019). Complex Tannins Isolated from Jelly Fig Achenes Affect Pectin Gelation through Non-Specific Inhibitory Effect on Pectin Methylesterase. *Molecules*, 24(8), 1601. https://doi.org/10.3390/molecules24081601.

Wang, N., Zhou, C., Basang, W., Zhu, Y., Wang, X., Li, C., Chen, L. & Zhou, X. (2021). Mechanisms by which mastitis affects reproduction in dairy cow: A review. *Reproduction in Domestic Animals*, 56(9), 1165-1175. https://doi.org/10.1111/rda.13953.

Zigo, F., Vasil, M., Ondrasovicova, S., Vyrostkova, J., Bujok, J. & Pecka-Kielb, E. (2021). Maintaining Optimal Mammary Gland Health and Prevention of Mastitis. *Frontiers in Veterinary Science*, 8. 10.3389/fvets.2021.607311.