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Identification of phenolic compounds and evaluation of antibacterial activity of *Pouteria splendens* fruit extracts

[Identificación de compuestos fenólicos y evaluación de la actividad antibacteriana de extractos de frutos de *Pouteria splendens*]

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Abstract: The escalating global problem of antibiotic resistance necessitates the exploration of novel antimicrobial sources, particularly from natural origins. This study examines the potential of *Pouteria splendens*, an endemic Chilean tree, as a source of antimicrobial agents. High-performance liquid chromatography (HPLC) analysis of fruit extracts revealed a rich composition of phenolic compounds, including phenolic acids, flavonoids, and stilbenes. The antibacterial activity of peel and pulp extracts was evaluated against a panel of clinically relevant Gram-positive and Gram-negative bacteria. *Escherichia coli* was the most susceptible bacterium to the pulp and peel extracts, which achieved a minimum inhibitory concentration (MIC) of 400 and 800 ppm, respectively. The presence of phenolic compounds like gallic acid, chicoric acid, kaempferol and resveratrol, known for their antimicrobial properties, may contribute to the observed activity. The results suggest that *P. splendens* fruit extracts are a promising natural antibacterial agent for the food industry.

Keywords: *Pouteria splendens*; Gallic acid; *Escherichia coli*; Chicoric acid; Fruit

Resumen: El creciente problema mundial de la resistencia a los antibióticos hace necesaria la exploración de nuevas fuentes de antimicrobianos, especialmente de origen natural. Este estudio examina el potencial de *Pouteria splendens*, un árbol endémico chileno, como fuente de agentes antimicrobianos. El análisis por cromatografía líquida de alta resolución (HPLC) de los extractos de frutos reveló una composición rica en compuestos fenólicos, incluidos ácidos fenólicos, flavonoides y estilbenos. Se evaluó la actividad antibacteriana de los extractos de cáscara y pulpa frente a un panel de bacterias Gram-positivas y Gram-negativas clínicamente relevantes. *Escherichia coli* fue la bacteria más susceptible a los extractos de pulpa y cáscara, que alcanzaron una concentración inhibitoria mínima (CIM) de 400 y 800 ppm, respectivamente. La presencia de compuestos fenólicos como ácido gálico, ácido chicórico, kaempferol, resveratrol y cianidina, conocidos por sus propiedades antimicrobianas, puede contribuir a la actividad observada. Los resultados sugieren que los extractos de fruta de *P. splendens* son prometedores agentes antibacterianos naturales para la industria alimentaria.

Palabras clave: *Pouteria splendens*; Ácido gálico; *Escherichia coli*; Ácido chicórico; Fruto

INTRODUCCIÓN

There are serious concerns about bacterial resistance, a significant issue for the public health and scientific communities. An epidemic caused by resistant bacteria is one of the challenges we are likely to face in the coming decades, prompting the search for new pharmaceutical alternatives, as inaction could lead to a future without therapeutic options (de Oliveira *et al*., 2022). In this search, plant-derived products are emerging as a promising alternative due to their availability, lower cost and, in many cases, fewer side effects compared to synthetic antibiotics (AlSheikh *et al.*, 2020; Murugaiyan *et al*., 2022).

Wide ranges of plants and their different parts, including roots, stems, flowers and fruits, are used as extracts for crude drugs, each of them being recognized for their varied medicinal properties (Al-Daihan & Bhat, 2012). Plants are recognized for their rich chemical biodiversity and therapeutic potential against resistant infections (Vaou *et al.*, 2021) and offer bioactive compounds such as phenolics. These secondary metabolites, ubiquitous in fruits, possess a wide range of biological properties, including remarkable antimicrobial activity against various pathogens (Rajkhowa *et al.*, 2024). For example, grape extracts are considered rich in polyphenols such as quercitrin and gallic acid, these compounds have been shown to be active against *Escherichia coli* and *Staphylococcus aureus*, including its methicillin-resistant strain. Polyphenols such as chlorogenic acid and ellagic acid, can be found in blackberries and blueberries extracts, and are able to inhibit *Listeria monocytogenes* and *Salmonella Enteritidis* (Lima et al., 2019). Therefore, the study of fruits as a source of phenolic compounds with antibacterial activity presents itself as a promising line of research.

Sapotaceae is a flowering plants family that is known for its richness in triterpenoid saponins, flavonoids, and polyphenolic compounds (Baky *et al.*, 2016; Baky *et al.*, 2022), and the only native species of this family in Chile is *Pouteria splendens* (A. DC.) Kuntze (syn. *Gayella splendens* (A. DC.) Aubrév), also known as "Palo Colorado" or "Chilean lúcumo", is an endemic tree species of Gondwanic origin, endemic to the mediterranean sclerophyll scrubland (Swenson *et al.*, 2023). This tree is found mainly in priority conservation sites such as the Federico Santa María cliffs (Valparaíso) and the Pichidangui-Los Molles area, where the largest population of the species resides (Sotes *et al.*, 2018). Its dispersal, historically dependent on extinct megafauna (Carvallo *et al.*, 2019), is now restricted. Although some native rodents act as surrogate dispersers, their efficiency is limited (Peña-Egaña *et al.*, 2018). It has been suggested that the rocky outcrops inhabited by *P. splendens* act as refuges for the regeneration of this tropical relict species, providing a favorable microclimate for seed germination and establishment (Carvallo *et al.*, 2019).

This tree is characterized by its whitish hermaphrodite flowers that give rise to a drupe-like, fleshy, yellow fruit that ripens to red, with a diameter of 2.5 to 3 cm (Henríquez *et al.*, 2012). Traditionally, its leaves are used in infusion in the treatment of gastrointestinal diseases and the bark is used in baths for joint ailments (Cordero et al., 2020). The fruits are consumed by local communities (Baeza, 1936), and although their nutritional contribution is valued, its potential as a source of bioactive compounds with potential antimicrobial activity has not been explored. Despite limited information, preliminary studies of the essential oil from the leaves of this species report the presence of compounds with antioxidant and antimicrobial properties such as globulol, phytol and nerolidol (Sotes *et al.*, 2006; Chan *et al.*, 2016; Lee *et al.*, 2016; Rodriguez et al., 2019). The main objective of this work was to identify the phenolic compounds present in ethanol extracts of *P. splendens* fruit peel and pulp by high performance liquid chromatography (HPLC) and to evaluate the antibacterial activity of the extracts against a panel of four clinically relevant bacterial strains, including three Gram-positive and one Gram-negative species, some of which show multidrug resistance.

MATERIAL AND METHODS

General

All chemical reagents purchased (Merck, Darmstadt, Germany or Aldrich, St. Louis, MO, USA) were of the highest commercially available purity and were used without previous purification.

Plant material

Fruits *P. splendens* were collected in January 2024 in "Punta Curaumilla", Laguna Verde bay, Chile. A voucher specimen (Ps-010124) was identified by forester engineer Patricio Novoa and deposited at the LPNSO laboratory, Universidad de Playa Ancha, Valparaíso, Chile.

Extracts by ultrasound

Ultrasound-assisted extraction was performed following a previously described method (Kumar *et al.*, 2021) with slight modifications. Briefly, 100 g of dried fruit pulp and peel powder was mixed with 200 mL of 96% ethanol and subjected to sonication for 1 h in an ultrasonic bath (Elma Schmidbauer GmbH, Germany) operating at 45 kHz, 800 W and 60°C. A constant temperature (± 3°C) was maintained by adding cold water to the bath. Filtration and concentration resulted in appreciable amounts of dry extract: 4.4 g of peel and 7.3 g of pulp. The dried extracts were stored in amber jars at 4°C until further analysis.

Identification of phenolic compounds by HPLC

To quantify and identify the phenolic compounds present in the extracts a HPLC-1100 Agilent equipped with a 3200 Q TRAP® hybrid quadrupole/linear ion trap mass spectrometer was used. To reconstitute the extracts of *P. splendens*, 5 mL of MeOH was used and made up to 10 mL with HPLC grade water. An aliquot was filtered through a 0.45 µm and aliquots of 20 µL were injected in the HPLC system. A Nucleosil C18 column (250 x 4.6 mm i.d., 5 µm particle size) was used and operated at 25°C. Mobile phase consisted of 0.1% formic acid (A) and methanol (100%) (B), at a flow rate of 0.5 mL/min. Elution gradient was 5-50% (B) from 0 to 30 min, followed by an increase to 75% B for 25 min (Tohma *et al.*, 2016). Mass spectra were obtained over a mass-to-charge ratio (m/z) of 100-1000 in full scan mode and the analyses were performed on positive and negative ionization modes. Peaks integration and MS spectra acquisition were performed with Thermo XcaliburTM Qualitative Browser (Thermo Xcalibur 2.2 SP1.48, Thermo Fisher Scientific Inc.).

Antibacterial activity test in vitro

Strains

Antimicrobial activity of the extracts was examined against a panel of bacteria, including the Gram-positive Strains *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), and *Listeria monocytogenes* (ATCC 19115), as well as the Gram-negative strains *Escherichia coli* (ATCC 8739). These pathogenic bacteria of clinical interest were cultured on Mueller-Hinton broth (MHB), incubated at 37°C, and subsequently stored at 4°C for further use. Microorganisms were supplied from Silob Chile (Silob Laboratorio Puerto Montt Limitada).

Antibacterial assay

The antibacterial activity of the extracts was determined using the serial microdilution method with modifications (Sandle, 2016) using sterile 96-well microplates. Samples were dissolved in ethanol and added to MHB medium at concentrations between 0 and 1600 ppm. Bacterial inocula were adjusted to achieve a concentration of $1 \cdot 10^6$ CFU/mL and a 1.5 µL aliquot was administered into the medium and incubated at 37°C on a shaker at 120 rpm. The minimum inhibitory concentration (MIC) was defined as the minimum extract concentration (ppm) at which visible bacterial growth was inhibited after 24 h of incubation. The percentage inhibition was calculated according to OD600 readings obtained by a spectrophotometer (Accu Reader M965), according to the method established by Barrera-Ruiz *et al*. (2020). Chloramphenicol and streptomycin were used as positive control, and MHB with 1% ethanol was used as negative control. The experiments were performed in triplicate.

Statistical analysis

The statistical analysis of recovery rates was performed by comparison within isolates and between the culturing media with a Student's t-test.

RESULTS AND DISCUSSION

Chemical composition

Analysis of both the peel and pulp of *P. splendens* fruit revealed a rich composition of phenolic compounds, including phenolic acids, flavonoids, stilbenes and other polyphenols (Figure No. 1). Some of the bioactive compounds identified are shown in Figure No. 2.

Fifty compounds were identified by RP-HPLC/MS, differentiated by retention time and molecular weight, verified with more than 1200 molecular weights found in the database Phenol-Explorer (Neveu *et al.*, 2010). The analysis identified a diverse range of polyphenols, with phenolic acids being the most abundant. Within this group, hydroxybenzoic acids, such as gallic acid, were prominent. Gallic acid is known for its numerous therapeutic activities, including anti-inflammatory, anti-cancer, antioxidant, and anti-angiogenic properties (Jasemi *et al*., 2022). Also present were hydroxycinnamic acids, notably chicoric acid. Research has demonstrated that chicoric acid

stimulates phagocytosis both *in vitro* and *in vivo*, inhibits hyaluronidase, protects collagen from free radical damage, and inhibits HIV-1 integrase (Yang *et al.*, 2022). A smaller proportion of flavonoids was also detected, including flavonols and flavones. Among these, kaempferol, known for its antioxidant, anti-inflammatory, antimicrobial, antidiabetic, and anticancer properties, was identified (Shahbaz *et al.*, 2023). Kaempferol also exhibits neuroprotective, anti-allergic, and cardioprotective effects (Kamisah *et al.*, 2023). Additionally, 6-hydroxyluteolin, which demonstrates *in vitro* antimalarial activity, was found (Kaur *et al.*, 2009). Furthermore, other significant phenolic compounds were identified, including the stilbene resveratrol, recognized for its antioxidant, anti-inflammatory, and neuroprotective properties (Gambini *et al.*, 2013). This study agrees with the characterization data of phenolic compounds present in *P. splendens* fruit collected in the Federico Santa Maria cliffs (unpublished data), revealing their bioactive potential.

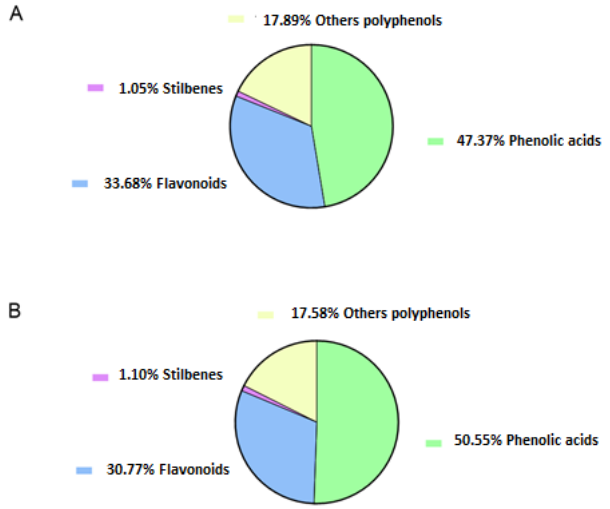

Figure No. 1
Percentage relative distribution of polyphenols presents in the extracts of *P. splendens* peel (A) and pulp (B)

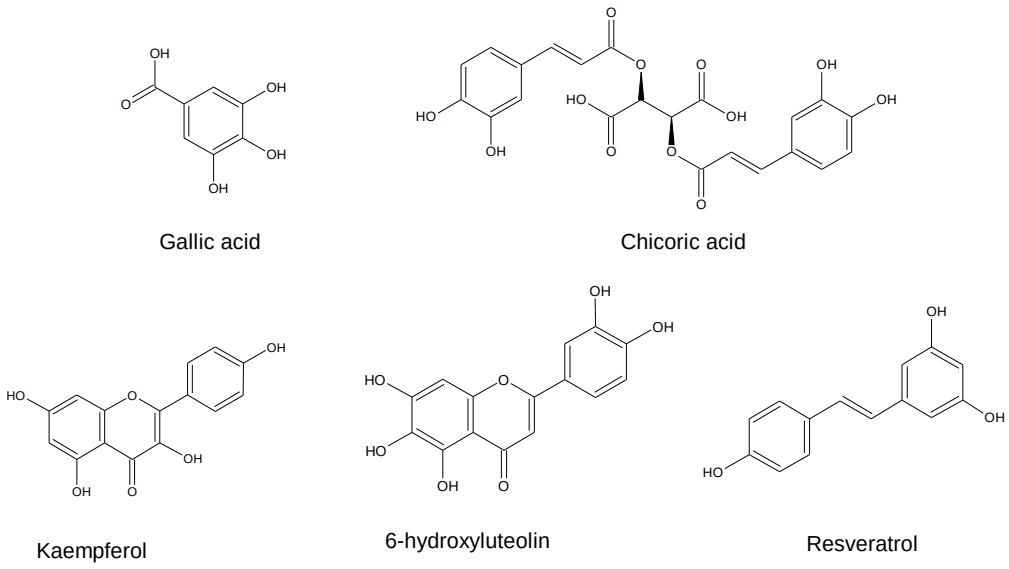

Figure No. 2
Biologically active compounds found in the pulp and peel extracts of *P. splendens*

Antibacterial Activity

Many pathogenic microorganisms have developed resistance due to inappropriate use of antibiotics. Therefore, there is a need to discover new substances from natural sources, including native fruits and their by-products like peel, which represent an important agricultural waste. In this study, antibacterial activity of extracts of *P. splendens* are given in Table No. 1 and Table No. 2.

Table No. 1
Percentage inhibition of bacterial growth of peel and pulp of *P. splendens* fruits extracts

Strains	Peel	Pulp
S. aureus	32.1%	77.3%
B. subtilis	64.4%	83.0%
L. monocytogenes	43.1%	74.0%
E. coli	62.0%	63.0%

Table No. 2
MIC (ppm) of peel and pulp of *P. splendens* fruits extracts

Strains	Peel	Pulp	Control A[a]	Control B[b]
S. aureus	1600	800	4	64
B. subtilis	1600	800	64	4
L. monocytogenes	1600	800	128	256
E. coli	800	400	8	4

The extracts were tested in triplicate and each value represents the mean of three experiments (n=3).
[a,b] Chloramphenicol and streptomycin were used as positive control, respectively

Compared to the pulp extract, the peel extract was less active, demonstrating higher MIC values (1600 ppm) against Gram-positive bacteria. However, the MIC value of peel extract against Gram-negative *E. coli* bacteria was significantly lower reaching 800 ppm. The pulp extract, conversely, exhibited a two-fold reduction in antibacterial activity against Gram-positive bacteria and even reached a MIC of only 400 ppm on *E. coli*. Overall, *E. coli* proved to be the most susceptible bacterial strain to the tested extracts. Although there are significant differences between the activity of the extracts under study and the antibiotics used as positive control, our results are distinguishable from other fruits extracts analysis.

For instance, these results outperform those obtained for *Pouteria venosa* by Dos Santos *et al.* (2014) which showed that its most active extract was able to inhibit microbial growth at concentrations between 1000 and 2500 mg/mL. Other fruits extracts from native trees from Africa such as *Piper gueenese*, *Aframomum melegueta* and *Xylopia aethiopica* presented a MIC of 5000 mg/mL for *E. coli*, and for *S. aureus* the results were close to 1000 g/mL (Ogbonna *et al.*, 2013). As mentioned, fruit extracts generally do not exhibit strong antimicrobial properties; however, pulp extracts from the fruit of *P. splendens* demonstrated a minimum inhibition value of 400 ppm, which is remarkable considering its fruit origin.

The antibacterial activity observed in *P. splendens* extracts against *E. coli* would be mainly justified by the presence of phenolic acids and flavonoids (Adamczak *et al.*, 2020). This activity could be related to the presence of several phenolic compounds with known antimicrobial activity, such as gallic acid, kaempferol, resveratrol and chicoric acid. Specifically, gallic acid, a hydroxybenzoic acid present in the extract, has demonstrated activity against *E. coli* by damaging the cell membrane and disrupting protein synthesis (Khare *et al.*, 2021). Kaempferol, flavonol identified in the extracts, has exhibited inhibitory activity against *E. coli*, possibly by inhibiting biofilm formation and altering bacterial membrane permeability (Periferakis *et al.*, 2022). Although to a lesser extent, resveratrol, a stilbene also present, has shown inhibitory effects on *E. coli* growth, especially in combination with other antibiotics (Mattio *et al.*, 2020), while chicoric acid is involved in the modulation of multiple cellular

processes, among them, it is suggested that one of the molecular targets of this molecule is the inhibition of NF-*k*b (nuclear factor kappa B), a protein complex involved in bacterial and viral infections by regulating DNA transcription (Peng *et al*., 2019). The presence of these compounds, acting synergistically, could explain the antibacterial activity detected in *P. splendens* pulp extracts against *E. coli*. While the detailed composition of the *P. splendens* extract in this study did not detect the presence of terpenes, we cannot rule out the possibility of their presence in undetectable concentrations that could also contribute to the observed activity. In fact, in the genus *Pouteria*, *Pouteria elegans* also demonstrated antimicrobial activity, but associated with the presence of monoterpenes such as α-pyrene (Aguiar *et al.*, 2019). Therefore, the synergy between phenolic compounds and the potential presence of terpenes, even at low concentrations, could be contributing to the observed antibacterial effect.

Therefore, the pulp and peel of the *P. splendens* fruit could be utilized in the future as a raw material for the development of a functional food or a natural antibacterial product for the food industry. Within the wellness concept, the following terms are coined: Health Care: Natural products aimed at self-care and improving well-being (Fuentes *et al.*, 2012). This is in line with the objective of research on native flora, which seeks to multiply little known and endangered species, evaluate their potential and promote sustainable management of the native forest, generating tools for their dissemination and preservation.

CONCLUSIONS

Pouteria splendens fruit extracts possess antibacterial activity and diverse phenolic compounds, particularly effective against *E. coli*. Although moderate, this activity warrants further investigation, including the isolation and characterization of bioactive compounds from leaves and bark. *P. splendens* shows promise as a source of novel antimicrobials for food preservation and combating antibiotic resistance, justifying future studies on mechanisms, efficacy, and optimized formulations across all plant parts.

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