Antimicrobial and antioxidant properties of the woody endocarp of native and commercial walnuts from Argentina

Propiedades antimicrobianas y antioxidantes del endocarpio leñoso de nogales nativos y comerciales de Argentina

Ingrid Georgina Orce [1], Sibila Ivonne Lencina [1, 3], Gabriela Inés Furque [2], Emilia Lorenzo [3], Gretel Rodriguez Garay [4], Fiamma Pereyra [3], María Rosa Alberto [5], Patricia Elizabeth Gomez Kamenopolsky [1, 3], Mario Eduardo Arena [5*]

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Abstract

Juglans australis is a tree from the *Juglandaceae* family found in the southernmost region of America. Its small edible nuts are not commercialized, and their bioactive characteristics are unknown. This study first reports the antioxidant, antiradical, and antibacterial activity of extracts from this native walnut against phytopathogenic bacteria and compared with its commercial counterpart, *J. regia* L. Different extracts from the woody endocarp (shells) were obtained using methanol and ethyl acetate. Methanolic extracts significantly inhibited phytopathogenic growth at all concentrations tested (0.1, 1, and 10 mg/mL). The best activity was reported against *Xanthomonas.* Highest total phenolics and the most significant antioxidant activity were determined in methanolic extracts (TPC: 121 mg gallic acid equivalent (GAE)/g of dried peel, FRAP: 58.6 mmol Trolox/100 g of peel dried and 9.7 mM Trolox/100 g of dried peel). Extracts from both species demonstrated congruent patterns. Gallic acid was the most abundant compound in the methanolic extract. However, extracts demonstrated superior efficiency, suggesting a potential synergistic effect among their components. Antioxidant and antimicrobial activity of methanolic extracts against *Xanthomonas* make them potential control agents.

Keywords
Juglans australis • phytopathogens • polyphenols • bioactive compounds • sustainable agriculture • *Xanthomonas* sp.

1 Centro Regional de Energía y Ambiente para el Desarrollo Sostenible. CREAS (UNCA-CONICET) Prado 366. San Fernando del Valle de Catamarca. CP K4700AAP. Catamarca. Argentina.
2 Universidad Nacional de Catamarca. Facultad de Ciencias Exactas y Naturales. Departamento de Química. Av. Belgrano al 300. San Fernando del Valle de Catamarca. CP K4700AAP. Catamarca. Argentina.
3 Universidad Nacional de Catamarca. Facultad de Ciencias Agrarias. Departamento de Química. Av. Maestro Quiroga 50. San Fernando del Valle de Catamarca. CP K4700AAP. Catamarca. Argentina.
4 Instituto Nacional de Tecnología Agropecuaria (INTA). Estación Experimental Agropecuaria Catamarca. Ruta 33 km 4.5 (4705). Valle Viejo. Catamarca. Argentina.
5 Instituto de Biotecnología Farmaceútica y alimentaria (INBIOFAL) Predio Universitario Ingeniero Herrera. Av. Kirchner 1900. CP 4000. San Miguel de Tucumán. Tucumán. Argentina. * mario.arena@fbqf.unt.edu.ar

Resumen

Juglans australis es un árbol perteneciente a la familia *Juglandaceae* que se encuentra en la región más austral del continente americano. Aunque las nueces también son comestibles, son pequeñas y no se comercializan, sus características bioactivas son desconocidas. Este estudio constituye el primer informe sobre la actividad antioxidante, antirradical y antibacteriana de extractos de la nuez nativa frente a bacterias fitopatógenas, y su comparación con la especie comercial, *J. regia* L. Se obtuvieron diferentes extractos a partir del endocarpio leñoso (cáscaras) utilizando metanol y acetato de etilo. El extracto metanólico resultó ser la fracción más activa e inhibió significativamente el crecimiento de los fitopatógenos en todas las concentraciones analizadas (0,1, 1 y 10 mg/mL). La mejor actividad se registró para el género *Xanthomonas*. El mayor contenido de fenoles totales y la actividad antioxidante más significativa se determinó en el extracto metanólico (TPC: 121 mg de ácido gálico equivalente (GAE)/g de cáscara seca, FRAP: 58,6 mmol de Trolox/100 g de cáscara seca y 9,7 mM de Trolox/100 g de cáscara seca). Los extractos de ambas especies se comportaron de manera similar. Al analizar la composición química, el ácido gálico fue el compuesto más abundante en el extracto metanólico. Sin embargo, los extractos mostraron una eficiencia superior, lo que sugiere un posible efecto sinérgico entre sus componentes. La actividad antimicrobiana de los extractos metanólicos contra *Xanthomonas*, junto con su capacidad antioxidante, resalta su potencial aplicación como agentes de control de fitopatógenos.

Palabras claves

Juglans australis • fitopatógenos • polifenoles • compuestos bioactivos • agricultura sustentable • *Xanthomonas* sp.

Introduction

Phytopathogens cause significant economic loss in agriculture (35). Even considering synthetic pesticides imply detrimental environmental and human health consequences, several products are still being used. In the last decade, eco-friendly compounds have emerged as alternative pesticides (8, 19). These natural antimicrobial agents are safer and cheaper than chemical agents (48) contributing to the economy and environment (9, 19, 44).

Several plant bioactive compounds have shown antimicrobial activity (14). Phenolic-rich plant extracts have shown significant activity against phytopathogens (22, 42), including *Xanthomonas* spp., a major threat to crops like rice and citrus, which have developed resistance to chemicals and antibiotics (25, 29). Some studies report the effects of extracts as natural antimicrobials against *Xanthomonas* sp. (3, 23, 24, 30).

Other pathogens, like *Clavibacter michiganensis,* are responsible for significant losses in tomato (32), and the bacterium *Erwinia amylovora* mainly affects pome fruit trees like pear, apple, quince, and loquat (5, 28). Carnaval *et al*. (2022) observed the inhibition effect of seriguela (*Spondias purpurea* L.) extract on *Clavibacter michiganensis* pv *michiganensis* and *Xanthomonas phaseoli.*

The genus *Juglans* includes over 20 species, being *J. regia* L. majorly significant due to its extensively studied nutritional and functional properties (7, 15). Walnut by-products, including shells, are rich in bioactive phytochemicals with antimicrobial potential for medicine, food preservation, and agroindustry (1, 4, 26, 27). Although their potential as biopesticides is recognized, the antimicrobial activity of these compounds is still unexplored. Meanwhile, shell biomass is often undervalued despite being a cost-effective, renewable resource.

On the other hand, *Juglans australis* is a native walnut tree from the Juglandacea family inhabiting the most austral region of South America, and the Northwestern subtropical rainforest in Argentina, locally known as "Yungas" (11), in Jujuy, Salta, Tucumán, La Rioja, and Catamarca (figure 1, page 189). Its fruit is an indehiscent, subglobose drupe with a thick, adherent mesocarp and a rigid shell (endocarp) containing the embryo (39) (figure 1, page 189). Contrasting with the extensive knowledge about commercial walnuts, information on this native species remains scarce. To date, no research reports antimicrobial or antioxidant activity of *Juglans australis*; except for its activity against herpes virus (37).

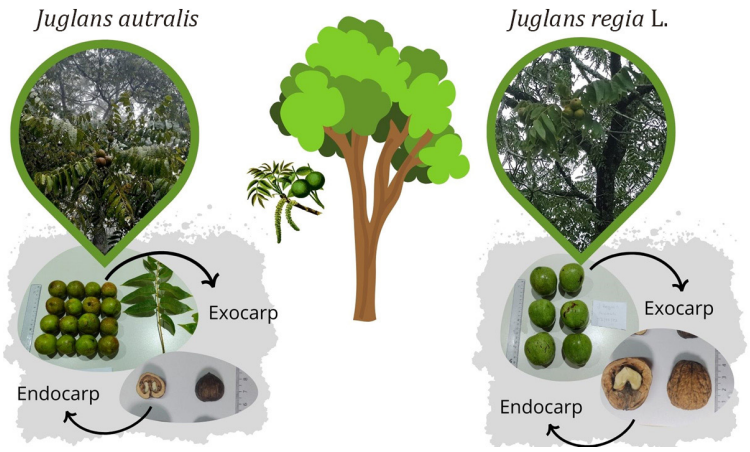


Figure 1. *Juglans australis* tree in Ancasti (Catamarca) city, Argentina (left) and *J. regia* (right). Morphological comparison of native *J. australis* and commercial *J. regia* L. fruits.

Figura 1 Árbol y frutos de la especie y *Juglans australis* (izquierda) y *Juglans regia* (derecha), correspondientes a la localidad de Ancasti, provincia de Catamarca, Argentina.

We aimed to analyze the antimicrobial and antioxidant properties of walnut shell extracts from the commercial *J. regia* and the native *J. australis* for future uses in agricultural management. These extracts would contribute to waste valorization while generating added value to the autochthonous species. Furthermore, extract chemical compositions allowed deeper comprehension of their bioactive activities.

Materials and methods

Sample collection

In 2021, samples of *J. regia* and *J. australis* walnut shells were collected in Ancasti, Catamarca, Argentina. Walnut shells were cleaned and dried under shaded conditions for a week. Selected samples were ground into small particles using a grinder.

Solvent extraction

Solvent extraction involved 50 g of the powdered walnut shells extracted with 250 mL of absolute methanol (MeOH) and ethyl acetate (AcOEt) for 45 min at room temperature and filtered through Whatman n° 4 (48). The solvents were evaporated under a vacuum in a Büchi R-210 rotavapor. The extracts obtained were redissolved in dimethyl sulfoxide (DMSO) to a final concentration of 0.1, 1, and 10 mg/mL and stored in the dark at 4°C for further use. All extractions were done in duplicate.

Determination of total phenolic content and antioxidant activities

Total polyphenol content (TPC) was determined colorimetrically using Folin-Ciocalteu's reagent at 765 nm. A standard curve was performed with gallic acid. The results were expressed as µg gallic acid/g dry weight (DW) (41). *In vitro* antioxidant activity was measured using the free radical elimination activity assay on 1,1, -diphenyl-2-picrylhydrazylradical (DPPH) (10), and ferric reduction capacity of plasma assay (FRAP) (6). Results are expressed in mmol Trolox equivalents/100 g dry weight (DW). All samples were analyzed in triplicate.

Identification of extract phenolic compounds by UHPLC-MS/MS

Phenolic compounds of extracts from walnut shells were identified by a UHPLC (Ultimate 3000 RSLC, Dionex - Thermo Scientific) equipped with a diode array detector and coupled to a TSQ Quantum ultra-triple-quadrupole mass spectrometer (TSQ Quantum Access Max, Thermo Scientific) and column Hypersil GOLD aQ (150 x 2.1 mm, 5 um)

(Thermo Scientific). The mobile phase was a binary mixture of solvents: mobile phase A corresponded to ultrapure water/formic acid solution (Merck, Darmstadt, Germany) (0.1% v/v), and mobile phase B corresponded to an acetonitrile / formic acid solution (Merck, Darmstadt, Germany) (0.1% v/v). Gradient conditions were as follows: 0-18 min, 97% A; 18-21 min, 90% B; 21-26 min, 97% A. Electrospray source of the MS was performed in negative mode. Eluate was monitored at 250 nm, flow rate was 0.3 mL min-1, injection volume was 15 µL, and the column was maintained at 35°C. Polyphenols were tentatively identified according to their retention times, UV/Vis spectra, high-resolution MS, and MS/MS spectra by comparison with pure compounds. We searched for gallic acid, cumaric acid, caffeic acid, ferulic acid, rutin, and eriocitrin (Sigma-Aldrich, St. Louis, MO, USA). The linearity of each calibration curve was confirmed by plotting the ratio of peak areas of phenolic compounds to the internal standard against compound concentration. Data were analyzed via LC-MS Xcalibur workstation software (Version 2.6, Thermo Fisher Scientific).

Antibacterial activity

Antibacterial activity of the different walnut shell extracts was evaluated against Gram-negative bacteria *Erwinia amilovora*, *Xanthomonas axonopodis* pv. *phaseolus,* and *X. campestris pv. campestris* 8004 and a Gram positive bacteria, *Clavibacter michiganensis*. Bacterial suspensions were prepared in Tryptic Soy Broth (TSB). By microdilution, microtiter plate wells were filled with bacterial suspension (10^7 CFU/mL) and the extract solution (final concentrations 0.1; 1 and 10 mg/ mL). Pure gallic acid, the main extract component, was incorporated in the antimicrobial assays at three different concentrations: 34 ppm (higher concentration detected in the extracts), 100 ppm, and 500 ppm. The extracts and gallic acid suspensions were prepared by diluting stock solutions in DMSO. Vehicle controls were prepared with DMSO and each bacterial culture. The 1% vehicle did not affect bacterial growth and was used as negative control. Streptomycin sulfate was also used as positive control. The microplates were incubated at 28°C for 24 h, and growth was detected using a microplate reader (Multiskan Go, Thermo) at 560 nm (45). Inhibition percentages were also calculated according to the Equation:

$$I\% = (Ac - As)/Ac) \times 100\%$$

where
Ac = absorbance of the control sample (phytopathogens without extract and antibiotic)
As = absorbance of phytopathogens + extract samples.

All assays were conducted three times and analyzed in triplicate.

Statistical analysis

ANOVA and Tukey test evaluated differences between treatments using INFOSTAT (Student version, 2020e).

Results and discussion

Total Phenolic compounds and Antioxidants activities

Table 1 (page 191), shows total phenolic content and antioxidant activity of organic extracts (ME: methanolic extract, EAE: ethyl-acetate extract) of *J. australis* and *J. regia*. Solvent extraction capacities varied significantly ($p < 0.05$). The highest phenolic content and reducing activity were observed in the methanolic extract corresponding to *J. australis* (121 mg GAE/g d.w. and 56.8 mmol Trolox/100 g d.w., respectively). However, the strongest antiradical activity was measured in the methanolic extract of *J. regia* ($p < 0.01$).

Tabla 1. Contenido fenólico total (TPC), actividades antirradicalias (DPPH) y reductoras (FRAP) de extractos metanólicos y de acetato de etilo obtenidos a partir del endocarpio de *J. australis* y *J. regia*.

Table 1. Total phenolic content (TPC), antiradical (DPPH) and reducing (FRAP) activities of methanolic and ethyl acetate of endocarp extracts from *J. australis* and *J. regia.*

Sample	Phenolic compounds (mg galic acid/g sample)	DPPH (mM Trolox/100 g DW)	FRAP (mM Trolox/100 g DW)
J. australis MeOH	121.1 ± 12.9 c	9.7 ± 0.6 b	56.8 ± 7.2 a
J. australis AcOEt	36.5 ± 5.4 a	2.06 ± 3 a	5.1 ± 0.2 b
J. regia MeOH	91.9 ± 13.4 b	11.5 ± 0.1 b	43.1± 0.6 a
J. regia AcOEt	39.9 ± 0.6 a	2.0 ± 0.1 a	13.8 ± 1.7 b

Value is expressed as mean ± standard error.
Different letters indicate statistically significant differences (p<0.05).
Los valores corresponden a la media ± error estándar.
Letras diferentes indican diferencias estadísticas (p<0,05).

Methanolic extracts had the highest total phenols content and the strongest antiradical and antioxidant activities. Extracts using polar solvents usually exhibit higher phenolic content and positively correlate with antioxidant potential (48). Among different factors in the extraction process, total phenolic compounds in walnut shell varies from 1 mg/g shell to 32.76 mg GAE mg/g shell (2, 26, 48). Here, we obtained 121 mg GAE mg/g shell in methanolic extracts of *J. australis*, significantly higher-more than two to ten times-than the reported (19).

When DPPH and FRAP activities were analyzed, methanolic extract of *J. australis* showed similar activity to *J. regia* and cited in the literature (42). Yang *et al.* (2014) analyzed the antioxidant and antiradical properties of walnut-shell extracts with different polarity solvents, demonstrating that methanol also shows the strongest antioxidant activity and reducing power. These established methods are reliable indicators of antioxidant potential in the native walnut, comparable with commercial *J. regia* activities, making it a potential source of bioactive compounds.

Solvent selection is crucial for antioxidant isolation by extraction methods. The chosen solvent significantly affects extract yield and its antioxidant activity due to the varying polarities of the extracted compounds (31). Methanolic and ethyl acetate extracts are commonly used in phytochemical studies. Methanol is a polar solvent that effectively extracts water-soluble compounds, including phenolics, flavonoids, and alkaloids. Ethyl acetate, on the other hand, is a less polar solvent that targets more lipophilic compounds, such as terpenes, steroids, and some fatty acids.

This difference in composition might depend on the genotype and environmental conditions during development, and maturity at harvest (2).

Identification and quantification of phenolic compounds

Phenolic compounds of walnut shell extract in *J. australis* and *J. regia* were determined using the UHPLC-MS/MS method. Results observed for *J. regia* extracts coincide with previous reports (2, 16, 18). The methanolic extract had the highest concentration of GLC (26.8 mg/L), followed by CMR (3.1 mg/L) and CFC (762 µg/L). Notably, rutin (RTN) was only detected in *J. regia* methanolic extract, albeit at a lower concentration (103 µg/L). In the ethyl acetate extract of *J. regia,* the most abundant were GLC (0.1 mg/L), followed by CMR (607 µg/L) and CFC (µg/L).

RTN in *J. regia* extracts suggests the potential unique properties of this species. Studies conducted on several parts of fruit and leaves consistently reveal that gallic acid is among the most abundant components in these extracts (2, 16, 18). Fernandez Argulló *et al.* (2021) recently reported that gallic, ellagic, and ferulic acids were the major phenolic compounds in walnut wood waste extracts. However, our study did not detect ferulic acid.

On the other hand, Gallic acid (GLC), caffeic acid (CFC), and cumaric acid (CMR) were identified in both *J. australis* extracts (methanolic and ethyl acetate) (table 2). The methanolic extract presents the highest GLC content (34000 µg/L), followed by CMR (672 µg/L) and CFC (351 µg/L). In ethyl acetate extracts, GLC was most abundant (9000 µg/L), followed by CMR (269 µg/L) and CFC (103 µg/L). Table 2 shows GLC content was significantly higher than CFC and CMR compounds for both extracts. Ours is the first characterization and quantification of phenolic compounds in this native walnut.

Table 2. Phenolic compounds in walnut shell extracts, and quantitative analysis of phenolic components in methanolic and ethyl acetate extracts of *J. regia* and *J. australis.*

Tabla 2. Compuestos fenólicos en extractos de cáscaras de nuez, y análisis cuantitativo del contenido de componentes fenólicos en extractos de acetato de etilo y metanólico presentes en *J. regia* y *J. australis*.

Extract	GLC (ppm)	CFC (ppb)	CMR (ppb)	RTN (ppb)
***J. australis* - MeOH**	34.2 ± 1.1	351 ± 46	672 ± 75	ND
***J. australis* - AcOEt**	9.2 ± 0.3	103 ± 10	269 ± 38	ND
***J. regia* - MeOH**	26.8 ± 0.6	762 ± 99	3.100 ± 0.6	103 ± 13
***J. regia* - AcOEt**	0.1 ± 0.0	26 ± 3	607 ± 45	ND

ppb = parts per billion = µg/L; ppm = parts per million = mg/L.

Note: AcOEt: ethyl-acetate; MetOH: methanolic; ppb = parts per billion = µg/L; ppm = parts per million = mg/L, GLC: gallic acid; CFC: caffeic acid; CMR: cumaric acid; RTN: rutin. ND: not detectable.

ppb = partes por billón = µg/L; ppm = partes por millón = mg/L.

Nota: AcOEt: acetato de etilo; MetOH: metanol; LOD: Límite de detección; ppb = partes por mil millones = µg/L; ppm = partes por millón = mg/L; GLC: ácido gálico; CFC: ácido cafeico; CMR: ácido cumárico; RTN: rutin. ND: no detectable.

Since studies on phenolic compounds are mainly conducted in *J. regia*, more information on *J. australis* extracts is needed. Considering differences between species, essential in-depth studies would allow understanding phytochemical profiles while identifying lost or gained compounds during crop domestication.

Antibacterial activity and gallic acid effects on bacterial growth

The effects of *J. regia* and *J. australis* extract and gallic acid (main compound in all extracts) were evaluated against phytopathogen growth (figure 2, page 193). All extracts tested showed the highest inhibition against *Xanthomonas* (figure 2 A and B, page 193). For *Xcc* 8004, methanolic extracts of *J. regia* (69.56%) and *J. australis* (72.31%) exhibited maximum inhibition at 10 mg/mL. Ethyl acetate extracts exhibited inhibitory activity against *Xcc* 8004, with *J. regia* demonstrating higher inhibition percentage (59.07%) than *J. australis* (41.61%).

All extracts from both *Juglans* sp. inhibited *Xanthomonas axonopodis* pv. *phaseoli*, exceeding 40% (figure 2B, page 193).

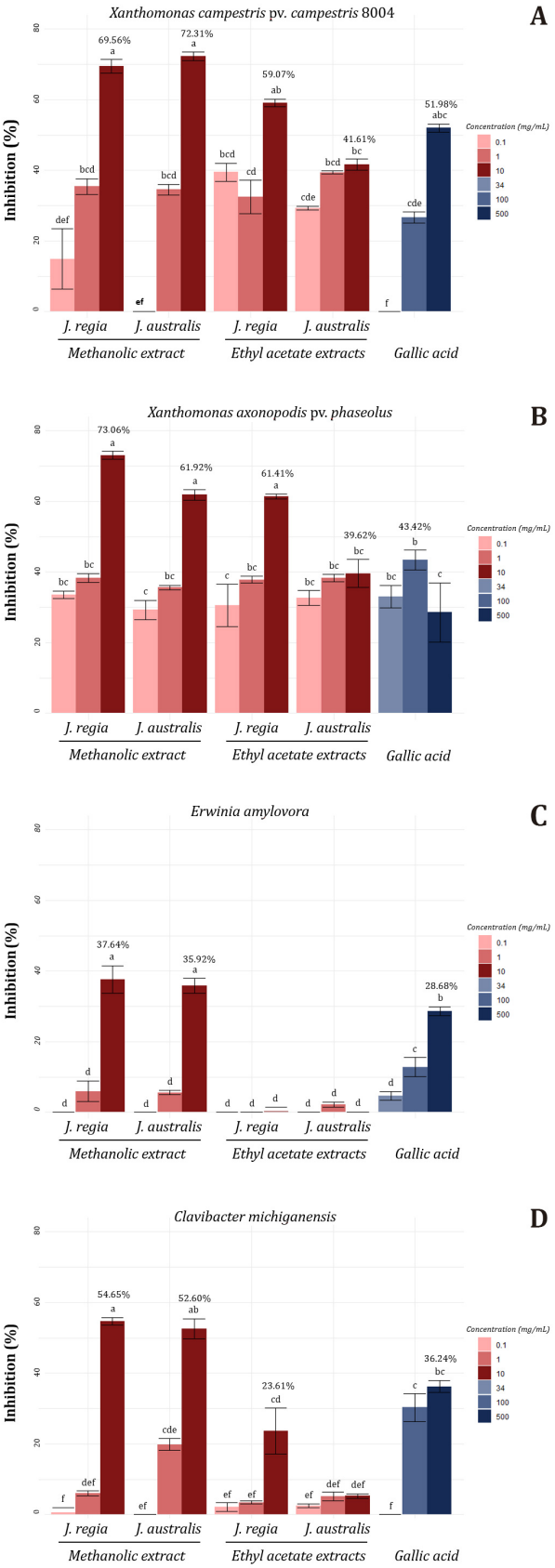
Considering *Erwinia amylovora*, methanolic extracts from both species diminished bacterial development in 37.64% (*J. regia*) and 35.92% (*J. australis*) at the highest tested concentration (figure 2C, page 193).

Once more, methanolic extracts at 10 mg/mL reached 54.65% and 52.60% inhibition against *Clavibacter michiganensis*, for *J. regia* and *J. australis*, respectively (figure 2D, page 193).

Gallic acid did not show substantial inhibition on the phytopathogens evaluated. At the highest concentration, it inhibited *X. axonopodis* (51.95%) (figure 2, page 193). Other studies mention antimicrobial activity of different parts of *J. regia* principally against pathogens of importance in human health (38), but none on antimicrobial effect against phytopathogens. Here, we observe that the methanolic extract from walnut shells had the best antimicrobial activity against the phytopathogens assayed even at the minimum concentration (0.1 mg/mL).

Shell extracts have inhibitory effects against *Xanthomonas* sp. and the highest amount of gallic acid (table 2), probably involved in antibacterial activities. Gallic acid is extensively studied, and its mechanism of action as an effective antimicrobial is well known (13, 21, 40).

Vu *et al.* (2017) report that gallic acid in walnuts can be found either in its free form or as part of hydrolyzable tannins. Nevertheless, gallic acid was less effective at inhibiting the phytopathogens assayed, suggesting a synergistic effect of the minor components.



Each value is expressed as mean ± standard error. Different letters indicate statistically significant differences ($p<0.01$).

Cada valor se expresa como media ± error estándar. Las diferencias estadísticamente significativas se indican con letras diferentes ($p < 0,01$).

Figure 2. Antibacterial activity of shell extracts from walnuts.

Figura 2 Actividades antibacterianas contra bacterias fitopatógenos de los diferentes extractos de cáscara de nuez.

Few studies have examined walnut shell extracts' antimicrobial effects, typically requiring higher concentrations (1-100 mg/mL) (31, 32, 44). Several reports used minimum bactericidal concentrations above 20 mg/mL for *J. regia* extracts (43) with notable activity against gram-negative bacteria like *E. coli* and *P. aeruginosa* (36). In our study, methanolic extracts effectively inhibited Gram-positive and Gram-negative phytopathogens at 10 mg/mL, particularly *Xanthomonas spp,* as previously reported with extracts from six walnut cultivars against Gram-positive (*Bacillus cereus, Bacillus subtilis*, *Staphylococcus aureus*) and Gram-negative bacteria (*P. aeruginosa, E. coli, Klebsiella pneumoniae*) (34).

Recently, the search for new natural compounds has gained interest given antioxidant and antimicrobial properties. Native plants are valued for economic and ecological benefits, with preservation playing a vital role (22).

The scarce information about using *J. australis* phytochemicals evaluates the effect of leaves and stem extracts on Herpes simplex virus (37). Here, we could demonstrate the efficacy of the native extracts over various phytopathogens.

Conclusion

In this study, we first report antibacterial activity of extracts from *J. australis* against phytopathogenic bacteria, and first findings on their particular antioxidant and antiradical activities. This contributions could enhance regional value. This research first characterizes walnut shell extracts from *J. australis*. Our findings demonstrate that methanolic extracts exhibit significant antimicrobial activity against *Xanthomonas* sp., suggesting natural biocontrol alternatives to copper-based formulations.

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