Pak. J. Bot., 58(4) April, 2026.

ARTICLE | DOI: http://dx.doi.org/10.30848/PJB2026-4(4)

ANTIOXIDANT AND ANTIFUNGAL EFFECTS OF *NERTERA GRANADENSIS* (Mutis ex L.f.) Druce AND HPLC-MS DETERMINATION OF ITS PHENOLIC COMPOUNDS

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Abstract

In this study the determination of biological effects of *Nertera granadensis* (Mutis ex L.f.) Druce, such as antioxidant and antifungal activities, was complemented with the qualitative analysis of phenolic compounds. It should be noted that the antioxidant and antifungal effects of *N. granadensis* have not been studied in previous studies. The antioxidant activities between four types of extracts obtained from the whole plant and the fruits were compared, and the antifungal effect of two types of extracts was assayed against the fungus *Rhizoctonia solani.* With the aim of evaluating the antioxidant effect, IC_{50} values were calculated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and total phenolic content was determined by means of the Folin-Ciocalteu method. The antioxidant effect was also determined as gallic acid equivalents for the DPPH assay and as Trolox equivalents for the ABTS assay. Phenolic compounds contained in the whole plant and fruits of *N. granadensis* were analysed using high-performance liquid chromatography and mass spectrometry (HPLC-MS) analysis. The extract obtained with methanol from the leaves, stems, and roots of *N. granadensis* using Soxhlet extraction (WPM) showed a higher antioxidant effect according to lower IC_{50} values of 2.8515 ± 0.0590 mg/mL and 0.7851 ± 0.0316 mg/mL, observed for the DPPH and ABTS assays, respectively. WPM also showed a fungistatic effect against *R. solani*. The results observed in this study revealed the antioxidant and antifungal effects of *N. granadensis*, and the HPLC-MS analysis allowed the identification of flavonols and hydroxycinnamic acid derivatives.

Key words: *Nertera granadensis*; Antioxidant effect; Total polyphenols; *Rhizoctonia solani*; Phenolic compounds; Liquid chromatography; Mass spectrometry

Abbreviations: A_0 = Absorbance of the negative control; A_1 = Absorbance of the samples; ANOVA = Analysis of variance; ABTS = 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH = 2,2-diphenyl-1-picrylhydrazyl; IC_{50} = Extract concentration needed to inhibit 50% of the absorbance; FFS = Extract obtained with methanol from the fresh fruits using Soxhlet extraction; FFM = Extract obtained with methanol from the fresh fruits using maceration; WPM = Extract obtained with methanol from the whole plant using Soxhlet extraction; WPW = Extract obtained with distilled water from the whole plant using Soxhlet extraction; W_1 = Dry mass of extract; GAE = Gallic acid contained in 1000 mg (1 g) of extract; HPLC-MS = High-performance liquid chromatography and mass spectrometry; HCADs = Hydroxycinnamic acid derivatives; IA (%) = Inhibition of the absorbance percentage; W_2 Initial mass of vegetal material; Y (%) = Percentage of mass of extract; PDA = Potato dextrose agar; TPC = Total polyphenol concentration; TE = Trolox contained in 1000 mg (1 g) of extract; DAD = UV–Vis diode array

Introduction

Plants produce a large group of organic compounds known as phytochemicals, which are provided with antioxidant activity and therefore capable of avoiding the harmful effects of free radicals (Nwozo *et al.*, 2023). The antioxidant activity exerted by natural antioxidants of vegetal origin could be an alternative to synthetic antioxidants for the prevention of oxidative stress (Tajini *et al.*, 2024). These phytochemicals, or natural antioxidants, consist of phenols, and the presence of phenols in plant material has been related to antimicrobial activity exerted by these kinds of bioactive molecules (Sciubba *et al.*, 2020). Moreover, antioxidants such as phenol compounds also have anti-inflammatory, anti-allergic, antithrombotic, antiviral, and anti-carcinogenic activities (Unsal *et al.*, 2021). Phenols represent a wide group of secondary metabolites and are characterised by one or more phenolic ring structures, which include flavonoids, lignans, and phenolic acids (Misra *et al.*, 2023). Another class of phenols or polyphenols, derived from flavonoids and phenolic acid, are tannins (Sharma *et al.*, 2021). The antifungal properties of some plant extracts have been related to the phenolic concentrations (Jara *et al.*, 2017). Indeed, it has been found that an increased antifungal effect in plant extracts is positively related to antioxidant activity and the content of phenolic compounds (Joaquín-Ramos *et al.*, 2020). Aspects to be considered for obtaining the plant extract are the extraction solvent (Wu *et al.*, 2022; Ting *et al.*, 2024) and the extraction method (Parafiniuk *et al.*, 2020), which are determining factors in the antioxidant

Received: 19-06-2025 | Revised: 25-10-2025 | Accepted: 05-11-2025 | Online: 19-12-2025

effect (Khursheed & Jain, 2021) and antifungal effect (Torun *et al.*, 2018). For example, in an extraction performed with six solvents, such as hexane, ethanol, ethyl acetate, acetone, methanol, and water, it was shown that the extracts obtained with methanol and ethanol presented the highest antifungal activities. In the ethanolic extract, chlorogenic acid, quinic acid, and catechin were identified, suggesting that the high inhibition percentage of mycelial growth of *Colletotrichum gloeosporioides* is due to the above-mentioned phenolic compounds (Vázquez-González *et al.*, 2020). The part of the plant used to obtain the plant extract is also a factor to consider for evaluating the biological activity of the plant extract (Gonzales *et al.*, 2023). The utilisation of plant extracts with antifungal activities, instead of chemical pesticides, means using sustainable and environmentally friendly methods against fungal diseases (Langa-Lomba *et al.*, 2021). The perennial herb *Nertera granadensis* (Mutis ex L.f.) Druce (Rubiaceae), a plant native to Chile, is distributed between the Coquimbo and the Magallanes regions and is also distributed on Juan Fernández Island. The common names of *N. granadensis* are coralito, chaquirita del monte, rucachucao, and coralillo (Rodriguez *et al.*, 2018). Its distribution around the Pacific Ocean has been described as unusually intercontinental and extensive, occurring in New Zealand and subantarctic islands (Tristan da Cunha), South America (Argentina, Bolivia, Chile, Colombia, Ecuador, Peru, and Venezuela), Central America (Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua, and Panama), North America (Mexico), Hawaii, Eastern Australia, Indonesia, Malaysia, Papua New Guinea, and the Philippines (Chen *et al.*, 2021a). This creeping plant is used for indoor ornamental purposes for no longer than a year, being difficult to maintain for a longer period of time. *Nertera depressa* is a synonym of *N. granadensis*, and this species has been employed for washing and cleaning wounds and ulcers by the Mapuche Amerindians of Chile (Houghton & Manby, 1985). To compare the antioxidant and antifungal activity in different extracts obtained from *N. granadensis*, different extraction methods were performed, such as maceration with methanol and extraction in a Soxhlet extractor with methanol and water. Methanol was considered according to the higher effects observed as described above when extracting with this solvent, and water was considered due to its polarity and non-toxic properties. The extractions were made from different plant parts, on the one hand from the leaves, stems, and roots, and on the other hand from the fruits. To complement the observed effects of the *N. granadensis* extracts, according to the relation of the antioxidant and the antifungal effects with the phenol compounds, a liquid chromatography-mass spectrometry analysis was carried out. It should be mentioned that studies about the antioxidant and antifungal effects of *N. granadensis* have not been previously performed.

Materials and Methods

Collection of vegetal material: The leaves, stems, roots, and fruits from *N. granadensis* were collected from a natural area in the Region of Araucania, Chile, South America (31°01′41″ S; 73°10′00″ O) (Fig. 1). After collection, the plant and the fruits were transported to the laboratory and were washed, dried, and kept in a cool and dry place. The fruits were stored under refrigeration.

Fig. 1. *N. granadensis* with fruits in its natural habitat before its collection.

Preparation of the extracts: The solutions, consisting of extracts from the fresh fruits of *N. granadensis*, were obtained through methanol Soxhlet (Glassco 3049/8) extraction (FFS) and a maceration process (FFM), as described below. The extraction time in the Soxhlet apparatus was performed until the solvent, in contact with 20.0 g of crushed fresh fruits, remained colourless. The maceration was done with a mixture of 20.0 g of crushed fresh fruits and 50 mL of methanol and was carried out over three weeks at room temperature. Moreover, the extracts of the whole plant, namely the leaves, stems, and roots of *N. granadensis*, were obtained with methanol (WPM) and distilled water (WPW) in a Soxhlet apparatus (Glassco 3049/8). Similarly, as described above, the extraction time was performed until the methanol or the water remained colourless. The mass of leaves, stems, and roots for obtaining the methanolic and aqueous extract was 70.0 g and 24.0 g, respectively. The four types of extracts mentioned above (FFS, FFM, WPM, and WPW) were treated in a rotary evaporator (Heidolph, Laborota 4001 efficient) at a bath temperature of 65°C ± 5°C with the aim of evaporating the solvent. The remnant of solvent in the extracts was eliminated in an oven at 35°C, and the dry extracts in glass vessels were stored at 4°C. The obtention of extract was calculated as a percentage of mass of extract (Y (%)), according to the dry mass of extract (W_1) and the initial mass of vegetal material (W_2). The Y (%) was obtained according to equation 1.

$$Y\ (\%) = W_1 \times 100 / W_2 \quad (1)$$

Therefore, the dry extracts from FFS, FFM, WPM, and WPW were used to prepare the solutions at determined concentrations, in mg of dried extract per mL of solvent, to perform the assays for antioxidant effect and the quantity of total polyphenols, described below.

Assays for antioxidant effect

Determination of antioxidant effect with the DPPH method: The assays of the antioxidant effect, performed with the DPPH radical to measure the absorbance diminution of DPPH, were determined in the presence of different

concentrations of the extracts. The antioxidant effects were expressed as the inhibitory concentration of each kind of extract concentration needed to inhibit 50% of the absorbance (IC_{50} value). The antioxidant capacities of the extracts were performed according to Chew *et al*., (2009), with modifications. The DPPH (Sigma-Aldrich) solution was prepared at a concentration of 0.036 mg/mL, dissolving DPPH in methanol. This solution was adjusted by adding methanol for reading an absorbance between 0.8 and 0.9 in a spectrophotometer (Thermo Scientific Genesys 10 UV) at a wavelength of 517 nm, using methanol for adjusting the zero point. For measuring the absorbance diminution of the DPPH solution, increasing concentrations of FFS, FFM, WPM, and WPW extracts in a volume of 175 µL were mixed with 2.0 mL of DPPH solution. The range of concentrations for each type of extract was established based on previous tests, assaying different concentrations to obtain absorbances between 0.1 and 0.9. The absorbance of the mixtures was read after 1 min of reaction at 517 nm in the spectrophotometer mentioned above, and the negative control consisted of a mixture formed by 175 µL of methanol and 2.0 mL of DPPH solution. The assays were done in triplicate. The antioxidant effect was calculated as an inhibition of the absorbance percentage (IA (%)) in accordance with equation 2.

$$IA\ (\%) = (A_0 - A_1 / A_0) \times 100 \quad (2)$$

Where A_0 is the absorbance of the negative control and A_1 is the absorbance of the samples.

The different ranges of concentrations of the solution and the extracts were assayed to obtain graphics of IA (%) versus concentrations, and the IC_{50} values were calculated based on the regression equations of these graphics. The antioxidant effect was also calculated as the gallic acid concentration, equivalent to the absorbance of an extract concentration, and the results were expressed as mg of gallic acid contained in 1000 mg (1 g) of extract (GAE). The equivalents performed in triplicate were calculated, interpolating the absorbance of an extract concentration in a graphic of gallic acid concentrations versus absorbance of 175 µL of increasing concentrations of gallic acid solution mixed with 2.0 mL of DPPH solution. To test the antioxidant effect during the time, kinetic assays in a spectrophotometer were performed, testing 175 µL of extract solutions mixed with 2.0 mL of DPPH solution. The kinetic assays were conducted in triplicate, considering the IC_{50} values determined in previous tests described above.

Determination of antioxidant effect with the ABTS method: As with the DPPH assay stated above, the absorbance diminution of the ABTS radical was assayed by mixing increasing concentrations of the methanolic or aqueous extracts obtained from *N. granadensis* with ABTS solution. The ABTS assay was performed with modifications, according to the proceeding of Kuskoski *et al*., (2004), for obtaining the ABTS radical cation as a product of the reaction between potassium peroxydisulfate (2.45 mM) (Merck) and ABTS (7 mM) (Sigma-Aldrich) in a volumetric flask, using the solvents water or methanol for the aqueous or methanolic extract, respectively, for determining each kind of extract's antioxidant effect. The ABTS radical cation was obtained at room temperature during 16 h in darkness. It was necessary to dilute the ABTS radical cation solution with water or methanol until an absorbance of 0.70 ± 0.02 at 734 nm, analysed in a spectrophotometer (Thermo Scientific Genesys 10 UV). A volume of 2.0 mL of the adjusted ABTS radical cation solution was mixed with 175 µL of increasing concentrations of FFS, FFM, WPM, and WPW extracts. After incubating for 1 min, the absorbance of the blend was read at a wavelength of 734 nm. The assays were performed in triplicate, and the negative control consisted of a mixture formed by 175 µL of water or methanol and 2.0 mL of ABTS radical cation solution. In order to obtain absorbances between 0.1 and 0.9, previous assays were carried out with different concentration ranges of extracts. The antioxidant effect was calculated in the same way as in the DPPH method, as an inhibition of the absorbance percentage (IA (%)) in accordance with equation 2. The antioxidant effect was also calculated as the Trolox concentration, equivalent to the absorbance of an extract concentration, and the results were expressed as mg of Trolox contained in 1000 mg (1 g) of extract (TE). The equivalents were calculated by interpolating the absorbance of an extract concentration in a graphic of Trolox concentrations versus absorbance of 175 µL of increasing concentrations of Trolox solution mixed with 2.0 mL of ABTS radical cation solution, according to the proceeding of Wakkumbura *et al*., (2020), with slight modifications, and the assays were performed in triplicate. Additionally, and considering the value of IC_{50} obtained from the graph of extract concentrations versus IA (%) kinetic assays were performed in triplicate.

Determination of total polyphenols: Total polyphenol concentration (TPC) in FFS, FFM, WPM, and WPW extracts was measured using the Folin-Ciocalteu assay as performed by Grzegorczyk-Karolak *et al*., (2015) with modifications. This method considers gallic acid as the phenol for expressing TPC as mg of gallic acid contained in 1000 mg (1 g) of FFS, FFM, WPM, and WPW. For calculating the TPC, the absorbance of an extract concentration was interpolated in a calibration curve of gallic acid considering increasing gallic acid concentrations (1, 2, 3, 5, and 6 µg/mL) versus absorbance measured at a spectrophotometer (Thermo Scientific Genesys 10 UV) at 765 nm. The Folin-Ciocalteu reagent is initially yellow, and in the presence of phenolic compounds, after a reaction of 30 min in darkness at room temperature, it turns blue. The mixture of gallic acid or the extracts and Folin-Ciocalteu (Merck) reagent (1.25 mL) was done by adding a sodium carbonate solution (5 mL, 20% m/v) and distilled water as the solvent in a 25 mL volumetric flask. The volume of a gallic acid solution or extract was calculated to obtain the required concentrations for each case. The assays for extract absorbance were performed in triplicate.

Qualitative chemical assays for the detection of natural product groups: To determine natural products contained in stems, fruits, leaves, and roots of *N. granadensis*, a general qualitative analysis was performed to identify groups of secondary metabolites. The different qualitative assays were done according to the metabolites assayed in the fruits and the WPM and WPW extracts. General

qualitative assays were performed to identify groups of alkaloids, flavonoids, saponins, tannins (Hussain *et al.*, 2011), coumarins (Sethy & Kullu, 2020), anthraquinones, and steroids (Kwodaga *et al.*, 2019). The result of each qualitative assay was expressed as a notorious presence of the metabolites (+++), a normal presence (++), or a low presence of the metabolites (+), and the non-existence of secondary metabolites was also noted (–).

Chromatographic assay for detecting the presence of phenolic compounds in WPM and FFS: Chromatographic assay of phenolic compounds was performed with a Shimadzu HPLC NEXERA system (Kyoto, Japan), equipped with a quaternary LC-30AD pump, a DGU-20A5R degasser unit, a CTO-20AC column oven, a SIL-30AC autosampler, a CBM-20A controller system, and a UV–Vis diode array (DAD). An SPD-M20A detector coupled in tandem with a QTrap LC/MS/MS 3200 Applied Biosystems MDS Sciex detector (Foster City, CA, USA). Instrument control and data collection were done by employing a CLASS-VP DAD Shimadzu Chromatography Data System and Analyst Software (version 1.5.2).

The chromatographic assay of flavonols and hydroxycinnamic acid derivatives (HCADs) of the WPM and FFS extracts was done according to a method previously described by Ruiz *et al*., (2013), with slight modifications. HPLC analyses were performed on a Kinetex C18 column (core-shell 150 × 4.6 mm, 2.6 µm) with a precolumn (Phenomenex, Torrance, CA, USA) and a binary mobile phase of 0.1% formic acid in water and acetonitrile at a flow rate of 0.5 mL/min with an injection volume of 10 µL. The mobile-phase gradient ranged from 15% to 25% acetonitrile for 14 min, from 25% to 35% for 11 min, from 35% to 100% for 1 min, from 100% to 15% for 1 min, and finally a stabilisation period of 10 min. The column temperature was adjusted to 40°C.

Identity assignment was done considering the retention times and by analysis of DAD and ESI-MS/MS spectra. The ionisation source was set in negative ionisation mode, controlling the following parameters: 5 V collision energy, 4000 V ionisation voltage, capillary temperature at 450°C, nebuliser gas at 40 psi, and auxiliary gas at 50 psi.

Antifungal effect of WPM and WPW: The antifungal effect of WPM and WPW was evaluated concerning the fungal growth of *Rhizoctonia solani* Kühn (BH-Rs-12), in accordance with the method of Elgorban *et al*., (2015), with slight modifications. The fungus was supplied by the Laboratorio de Biotecnologia de Hongos belonging to the Universidad de Concepción. Before performing the antifungal assay, the fungus was activated at 24ºC in a stove (Binder ED53) for 72 h on the potato dextrose agar (PDA) culture medium (Liofilchem 610102). From WPM and WPW aqueous solutions with concentrations of 15, 20, and 25 mg/mL, aliquots of 1 mL were mixed with 19 mL of sterile PDA in sterile plates to obtain WPM concentrations of 0.75, 1.0, and 1.25 mg/mL. The negative control was composed of 19 mL of sterile PDA and 1 mL of sterile water. Once the mixtures were cooled, a piece of PDA with the fungus hyphae of 1 cm in diameter was placed in the middle of each plate. The fungal growth was determined by measuring the growth of the halo vertically and horizontally. The assays were carried out in triplicate.

Statistical Method

Results were calculated as means ± standard deviation (SD). For each measured data point, quantitative differences between organs were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. $DPPH^{-1}$ transformation was used, given the non-compliance with the assumption of homoscedasticity (DPPH variable). When assumptions were not fulfilled (GAE and TE variables), the nonparametric Kruskal-Wallis ANOVA and multiple comparisons Dunn test were applied (Conover, 1999). The STATISTICA 10.0 version 7® program was used. The level of statistical significance was established at $p<0.05$.

Results and Discussion

The extracts obtained from *N. granadensis* were tested to evaluate antioxidant effect and polyphenolic content, to detect groups of secondary metabolites, to identify phenols, and to determine antifungal effect.

Mass of the extracts and percentage yields (Y(%))

Four modalities of extracts were obtained from *N. granadensis*:

I. Extract obtained with methanol from the fresh fruits using Soxhlet extraction (FFS).
II. Extract obtained with methanol from the fresh fruits using maceration at room temperature (FFM).
III. Extract obtained with methanol from the whole plant using Soxhlet extraction (WPM).
IV. Extract obtained with distilled water from the whole plant using Soxhlet extraction (WPW).

The Y (%), calculated from the mass of the extract and the mass of the plant material, were 3.60% (FFS), 3.65% (FFM), 12.10% (WPM) and 23.79% (WPW). The extraction method employed to obtain fruit extracts did not notoriously influence the yield of extraction. Comparatively, the yields obtained from the whole plant were higher than the yields obtained from the fruits, using water as the extraction solvent.

Antioxidant effect

Antioxidant effect of FFS, FFM, WPM and WPW evaluated through the DPPH and ABTS methods: The different IC_{50} values measured by the DPPH method of the four extracts revealed differences in the antioxidant effect. The IC_{50} values calculated from the equations resulting from the graphics of extract concentrations versus DPPH absorbance are shown in Table 1, and considering these values, it is possible to deduce that the WPM extract obtained with methanol from the whole plant has a greater antioxidant effect, as reflected in the significantly lower IC_{50} value of 2.8515 ± 0.0590 mg/mL, and contrary to the FFM extract obtained also with methanol from the fruits, demonstrated the lowest antioxidant effect with the significantly higher IC_{50} value of 18.1771 ± 5.7363 mg/mL. The extraction method carried out with the Soxhlet extractor was more effective at extracting secondary metabolites with an antioxidant effect, according to the

observed IC_{50} values of the FFS, WPM, and WPW extracts. When comparing the IC_{50} values of the FFS and WPW extracts, no statistically significant differences were observed. It should also be noted that in our study the radical scavenging for DPPH was registered after just one minute of oxidation-reduction reaction, and this briefer reaction time compared with other studies, in which the lower IC_{50} values were measured, could be related to the radical scavenging registered after 30 min of oxidation-reduction reaction (Manojj *et al.*, 2020; Thy *et al.*, 2024). In other words, other studies require lower concentrations of extracts because the radical scavenging increases over time (Schneider *et al.*, 2024).

According to the results obtained for IC_{50} values according to the ABTS method, differences in the antioxidant effect were also observed, as in the case of the different IC_{50} values observed in accordance with the DPPH method. The IC_{50} values were calculated from the equations resulting from the graphics of extract concentrations versus ABTS absorbance and are presented in Table 2. As in the DPPH method, the FFS, WPM, and WPW extracts, obtained from the Soxhlet extractor, showed significantly lower IC_{50} values in comparison with a significantly higher IC_{50} value of 3.0597 ± 0.3263 mg/mL, confirming the Soxhlet extraction as a more effective method for extracting secondary metabolites with an antioxidant effect than the maceration method. When comparing the significantly different values of IC_{50} between WPM and WPW extracts, measured by the DPPH and ABTS methods, it should be inferred that methanol is more effective than water for extracting secondary metabolites with an antioxidant effect. The above-mentioned difference between methanolic and aqueous extracts has also been studied in other species (Elmi *et al.*, 2020; Nipun *et al.*, 2021). Nevertheless, in other species, considering the IC_{50} values, a higher antioxidant effect has been detected in aqueous extract obtained from aerial parts and roots of *Chaerophyllum bulbosum* (Tel-Çayan *et al.*, 2022). As well as with our study, in a previous study of the herb *Galium aparine* L. (Rubiaceae), when comparing Soxhlet extraction with maceration and using methanol, the extract obtained in a Soxhlet extractor showed a higher antioxidant effect in relation to the extract obtained with maceration, according to DPPH and ABTS methods (Özmatara, 2021). In the same way as for the DPPH method, the radical scavenging for ABTS was registered after one minute of reaction of each extract and ABTS. Consequently, higher concentrations of extracts than in other studies were applied, in which the antioxidant effect was registered after 6 min (Ahmad *et al.*, 2022), 10 min (Chen *et al.*, 2021b) and 20 min (Abbassi *et al.*, 2024) of reaction.

As shown in the following Fig. 2(a, b, c, d), all extracts exerted an increasing antioxidant effect during the testing time. The antioxidant effect tested with DPPH and ABTS remained stable after reaching higher levels of antioxidant effect, as observed in the above-mentioned figure.

The equivalents of gallic acid were performed in triplicate with DPPH and calculated as mg of gallic acid contained in 1.0 g of each kind of extract (GAE), and the corresponding calibration curve is shown in Table 3. The antioxidant effect expressed as GAE was significantly higher in the WPM extract in comparison with the significantly lower GAE value observed in the FFM extract, and this statistically significant difference is related to lower IC_{50} values calculated by the DPPH and ABTS methods, observed in the WPM extract.

The ABTS assay also permitted the expression of equivalents in a molecule with an antioxidant effect. The results, expressed as mg of Trolox contained in 1.0 g of the extracts (TE) and calculated from the calibration curve, are presented in Table 4. Based on the significantly different results of TE for the WPM and WPW extracts, it can be inferred that, when compared to an aqueous extraction, a methanol extract allows the obtention of more secondary metabolites with an antioxidant effect, similar to that of Trolox.

Concentration of polyphenols: The content of total polyphenols expressed as mg of gallic acid contained in 1.0 g of each kind of extract is presented in Table 5, and the total polyphenols' concentration (TPC) was calculated from the respective calibration curve. The TPC detected in the FFM extract, obtained by using the maceration method, was significantly lower than those obtained by using the Soxhlet extractor, and this result is connected with the significantly higher IC_{50} value observed in the FFM extract, according to the results informed for the DPPH and ABTS methods. Conversely, by linking the significantly higher TPC in the WPW extract in relation to TPC in the WPM extract, there is no relation with the above-mentioned IC_{50} values. In fact, the IC_{50} values observed in the WPW extract for the DPPH and ABTS methods were significantly higher than those observed in the WPM extract.

General analysis of secondary metabolites: The results of preliminary qualitative analysis revealed differences between the content of natural product groups in fruits and in the WPM and WPW extracts. In fact, a marked presence (+++) of coumarins in WPM extract and of tannins in WPW extract was detected. It can be inferred that the coumarins detected in the WPM extract could be coumarin phenolic compounds (Todorov *et al.*, 2023) that could have a significantly higher antioxidant effect, obtained in accordance with the IC_{50} values, for the DPPH and ABTS methods. Tannins are complex polyphenols with strong polarities (Li *et al.*, 2024), and their presence in WPW extract can be attributed to water, used as the extraction solvent (Murali *et al.*, 2021). The presence of the above-mentioned polyphenols may have had an impact on the significantly higher TPC value of the WPW extract. Additionally, a marked (+++) presence of saponins and steroids was observed in both WPM and WPW extracts. On the other hand, a marked (+++) presence of alkaloids, saponins, and tannins was detected in the fruits.

Phenolic compounds in WPM and FFS: As can be observed in Fig. 3, the WPM and FFS extracts presented a higher proportion of phenolic compounds that are detected at 320 nm, such as HCADs, and a much lesser proportion of phenolic compounds that absorb at 360 nm, such as flavonols. At other characteristic wavelengths, there were no important signals for other phenolic compounds.

In Table 6, the main HCADs found with the HPLC-MS/MS analysis were caffeoylquinic acid isomers, with traces of coumaric acid derivate. Only traces of flavonol glycosides were observed, with myricetin being the most prominent in the WPM extract. The same profile was detected in the FFS extract, but in a much lesser proportion.

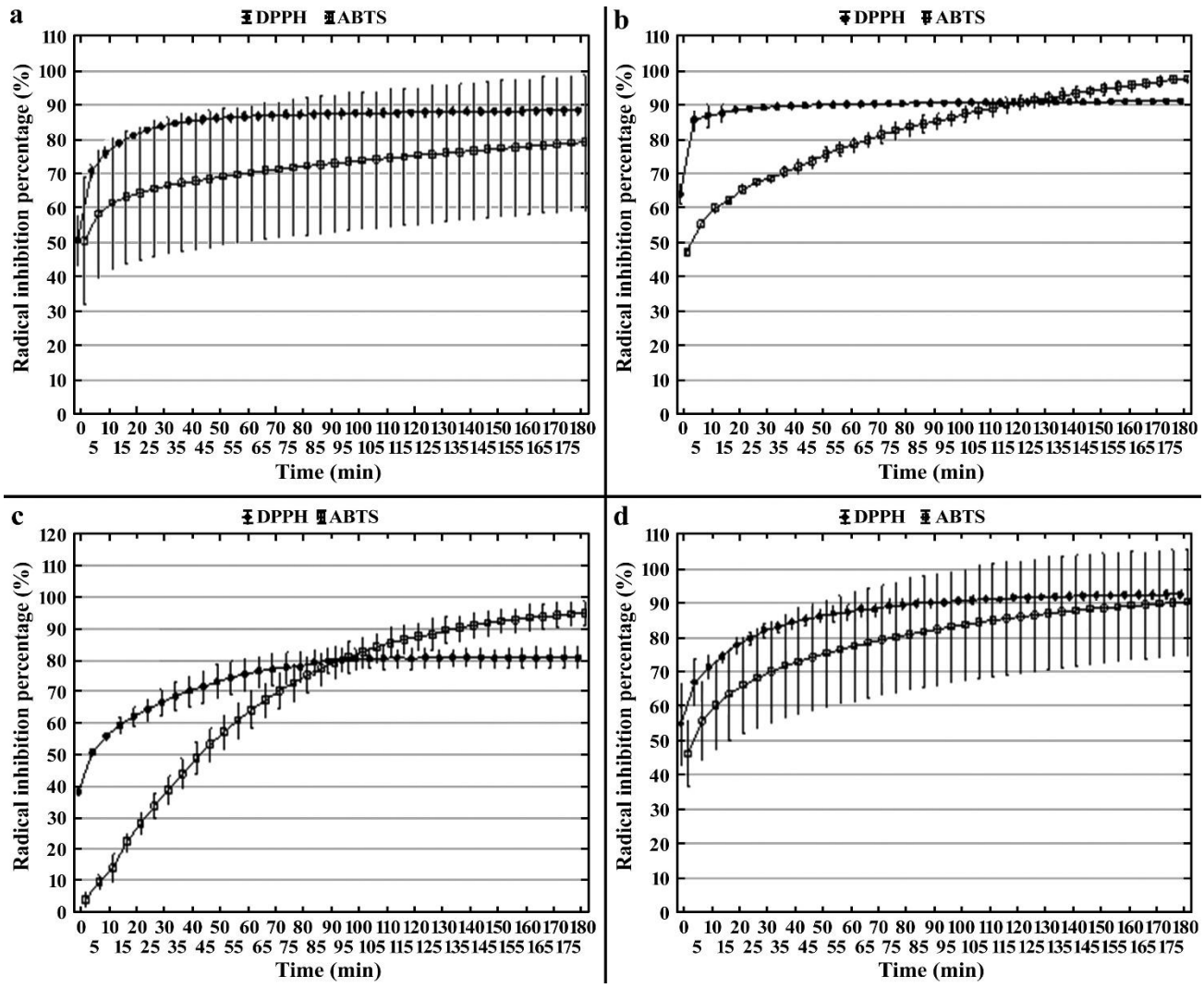

Fig. 2. Applying on all IC_{50} concentrations: **a**, Antioxidant effect of FFS over 3 h; **b,** Antioxidant effect of FFM over 3 h; **c,** Antioxidant effect of WPM over 3 h; **d,** Antioxidant effect of WPW over 3 h.

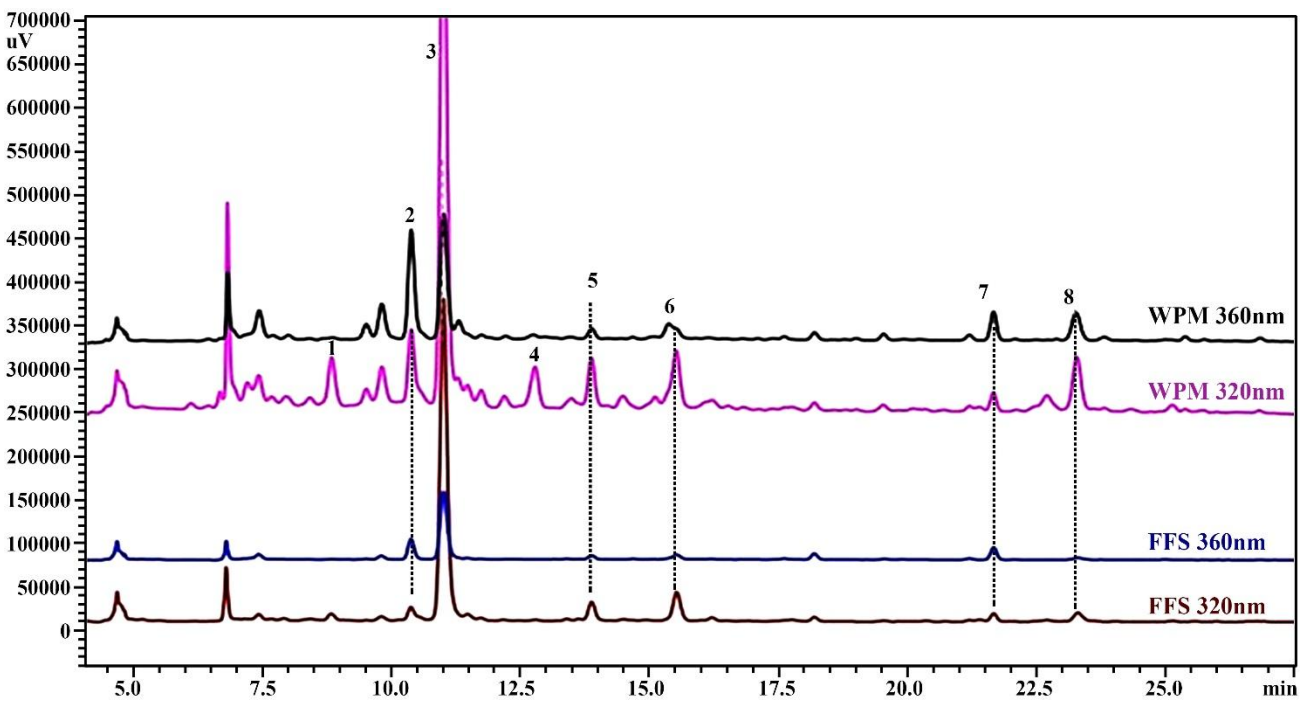

Fig. 3. Chromatograms of WPM and FFS *N. granadensis* extracts registered at wavelengths of 360 nm and 320 nm for flavonoids and for HCADs, respectively.

Table 1. Antioxidant effects of FFS, FFM, WPM, and WPW, obtained from the DPPH assay, informed by means of IC_{50} values.

Extract	Replicates	Equation	IC_{50}	IC_{50} (av. ± S.D.)
FFS	R_1	$y = 0.0223x^3 - 0.7716x^2 + 10.645x - 2.8608$	10.6896	8.8458 ± 1.9142^{b} mg/mL
	R_2	$y = 0.0042x^3 - 0.2846x^2 + 7.8383x - 0.4777$	8.9796	
	R_3	$y = 0.0149x^3 - 0.6912x^2 + 11.646x - 2.2096$	6.8683	
FFM	R_1	$y = 17.318\ln(x) - 5.5685$	24.7472	18.1771 ± 5.7363^{a} mg/mL
	R_2	$y = -0.01x^2 + 3.2204x + 2.1359$	15.6205	
	R_3	$y = 0.0046x^2 + 3.1585x + 4.3414$	14.1636	
WPM	R_1	$y = 14.66x + 8.3767$	2.8392	2.8515 ± 0.0590^{c} mg/mL
	R_2	$y = 15.667x + 6.1387$	2.7996	
	R_3	$y = 15.485x + 4.8507$	2.9157	
WPW	R_1	$y = 5.5166x - 0.4879$	9.1519	9.3389 ± 0.4578^{b} mg/mL
	R_2	$y = 5.5578x - 0.0431$	9.0041	
	R_3	$y = 4.8556x - 2.1207$	9.8606	

Values in each column combined with different letters are significantly different based on the results obtained in accordance with the Tukey test ($p<0.05$)

Table 2. Antioxidant effects of FFS, FFM, WPM, and WPW, obtained from the ABTS assay, informed by means of IC_{50} values.

Extract	Replicates	Equation	IC_{50}	IC_{50} (av. ± S.D.)
FFS	R_1	$y = -9.0575x^2 + 58.566x - 2.7727$	1.0822	1.2185 ± 0.2277^{bc} mg/mL
	R_2	$y = -10.684x^2 + 66.4x - 9.7623$	1.0919	
	R_3	$y = -5.5192x^2 + 49.414x - 11.086$	1.4813	
FFM	R_1	$y = -1.4427x^3 + 10.53x^2 - 2.847x + 9.3095$	2.7005	3.0597 ± 0.3263^{a} mg/mL
	R_2	$y = 2.1219x^2 + 9.5652x - 0.9711$	3.1407	
	R_3	$y = 2.0738x^2 + 8.3825x - 1.0841$	3.3378	
WPM	R_1	$y = 52.995x + 6.8457$	0.8143	0.7851 ± 0.0316^{c} mg/mL
	R_2	$y = 64.15x - 0.6486$	0.7895	
	R_3	$y = 68.289x - 1.32$	0.7515	
WPW	R_1	$y = 5.4252x + 43.988$	1.1082	1.5048 ± 0.3504^{b} mg/mL
	R_2	$y = 6.1585x + 39.939$	1.6337	
	R_3	$y = 6.034x + 39.305$	1.7725	

Values in each column combined with different letters are significantly different based on the results obtained in accordance with the Tukey test ($p<0.05$)

Table 3. Antioxidant effect of FFS, FFM, WPM, and WPW expressed in terms of GAE.

Extract	Equation of the calibration curve	Replicates	GAE*	GAE* (av. ± S.D.)
FFS	$y = 1.9362x^{-0.517}$	R_1	2.5271	3.1538 ± 1.0328^{ab} mg/g
		R_2	4.3458	
		R_3	2.5885	
FFM	$y = 1.9362x^{-0.517}$	R_1	1.9850	1.6540 ± 0.2957^{b} mg/g
		R_2	1.5609	
		R_3	1.4161	
WPM	$y = 0.0002x^2 - 0.0249x + 0.9711$	R_1	5.3980	5.0605 ± 0.2929^{a} mg/g
		R_2	4.9118	
		R_3	4.8718	
WPW	$y = 0.0082x^2 - 0.154x + 0.6262$	R_1	3.7302	3.7344 ± 0.0598^{ab} mg/g
		R_2	3.6769	
		R_3	3.7962	

*The expression of the result is mg of gallic contained in 1000 mg (1 g) of FFS, FFM, WPM, and WPW. Values in each column combined with different letters are significantly different based on the results obtained in accordance with the Dunn test ($p<0.05$)

Antifungal effect: The extract WPM demonstrated a fungistatic effect against the fungus *R. solani*, in accordance with the observed inhibition of the fungal growth (Fig. 4). In fact, the diameter of the growth area decreased more with the maximum extract concentration of 25 mg/mL and 1.25 mg/mL assayed in each plate. In addition, the fungistatic effect was increased during the time, observing a maximal effect at 96 h (Fig. 5). It should also be mentioned that after diluting 1 mL of the concentrated extract in 19 mL of PDA, the effective concentrations of WPM in the test plates were 0.75, 1.0, and 1.25 mg/mL. The coumarins detected as a group of secondary metabolites, only in the WPM extract, could be related to the fungistatic effect of the WPM extract,

according to a previous study about the antifungal effect of coumarins against *R. solani* (He *et al.*, 2021). Further research had revealed the antifungal effect of coumarins. The coumarin scopoletin exhibits a strong effect against the necrotrophic fungus *Alternaria alternata* (tobacco pathotype), and scopoletin biosynthesis is activated in tobacco leaves in the presence of the fungus (Sun *et al.*, 2014). A metabolic response, consisting of the biosynthesis of the coumarin basic skeleton, was observed in the leaves of *Heracleum moellendorffii* infected with *Erysiphe heraclei*, known as powdery mildew (Liu *et al.*, 2024). The HCADs observed in the WPM extract by HPLC-DAD-ESI-MS/MS were principally caffeoylquinic acid isomers, and artichoke leaf extracts, also containing these kinds of polyphenols, exhibited a significant antifungal effect against the phytopathogenic fungus *Alternaria alternata* (Masci *et al.*, 2024). According to HPLC-DAD-ESI-MS/MS, in the WPM extract, the heterosides of the flavonols myricetin, quercetin, and isorhamnetin were also detected, and all these flavonols have an antifungal effect (Al Aboody & Mickymaray, 2020; Tian *et al.*, 2021). No antifungal effect was observed after carrying out the antifungal assay with the WPW extract.

Table 4. Antioxidant effect of FFS, FFM, WPM, and WPW expressed in terms of TE.

Extract	Equation of the calibration curve	Replicates	TE**	TE** (av. ± S.D.)
FFS	y = - 0.0112x + 0.6563	R_1	25.7436	26.5812 ± 0.8502^{ab} mg/g
		R_2	27.4435	
		R_3	26.5566	
FFM	y = - 0.0112x + 0.6563	R_1	10.4572	8.3103 ± 1.8593^{ab} mg/g
		R_2	7.2230	
		R_3	7.2507	
WPM	y = 0.00005x^2 – 0.0127x + 0.5883	R_1	31.2764	34.8054 ± 3.8384^{a} mg/g
		R_2	34.2476	
		R_3	38.8921	
WPW	y = -0.0434x + 0,6402	R_1	4.8640	4.7748 ± 0.0975^{b} mg/g
		R_2	4.7897	
		R_3	4.6707	

** The expression of the result is mg of Trolox contained in 1000 mg (1 g) of FFS, FFM, WPM, and WPW. Values in each column combined with different letters are significantly different based on the results obtained with the Dunn test ($p<0.05$)

Table 5. Polyphenol content in FFS, FFM, WPM, and WPW.

Extract	Equation of the calibration curve	Replicates	TPC***	TPC*** (av. ± S.D.)
FFS	y = 0.1128 – 0.1033	R_1	37.1587	36.9814 ± 0.2032^{b} mg/g
		R_2	36.7597	
		R_3	37.0257	
FFM	y = 0.1128 – 0.1033	R_1	22.3981	22.4690 ± 0.4211^{d} mg/g
		R_2	22.9210	
		R_3	22.0878	
WPM	y = 0.118 + 0.035	R_1	29.4110	29.6624 ± 0.3038^{c} mg/g
		R_2	30.0000	
		R_3	29.5763	
WPW	y = 0.1109 + 0.0178	R_1	81.4248	80.8988 ± 0.4693^{a} mg/g
		R_2	80.7485	
		R_3	80.5230	

*** The expression of the result is mg of gallic acid contained in 1000 mg (1 g) of FFS, FFM, WPM, and WPW. Values in each column combined with different letters are significantly different based on the results obtained with the Tukey test ($p<0.05$)

Table 6. Identification of major flavonoids and HCADs in WPM and FFS *N. granadensis* extracts analysed with HPLC-DAD-ESI-MS/MS.

N° peak	Identifications	t_R (min)	DAD (nm)	[M-H]$^-$	Fragments
1.	Caffeoylquinic acid isomer	8.33	324	535	191
2.	Caffeoylquinic acid isomer	10.9	324	553	191
3.	Caffeoylquinic acid isomer	11.1	324	553	191
4.	Coumaric acid derivate	12.5	284	325	163, 119
5.	Myricetin-3-galactoside (*)	13.8	340	479	317, 287, 271
6.	Myricetin-3-glucoside(*)	16.05	360	479	317, 287, 271
7.	Quercetin-3-glucoside(*)	21.12	360	463	301, 271, 179, 163
8.	Isorhamnetin hexoside	23.67	351	477	315, 285, 299, 271

*Identification verified with commercial standards

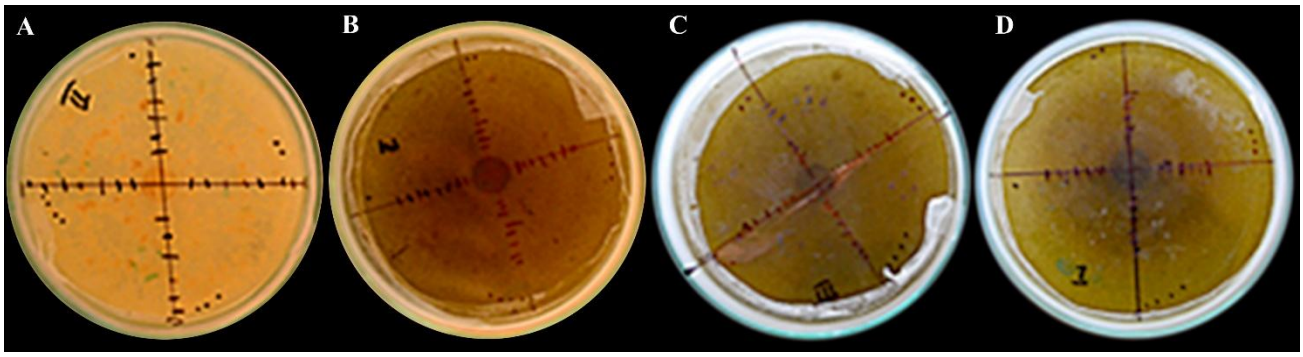

Fig. 4. Antifungal effect of the WPM extract against *R. solani* by increasing concentrations of WPM: a.) negative control, b.) 15 mg/mL, c.) 20 mg/mL, d.) 25 mg/mL.

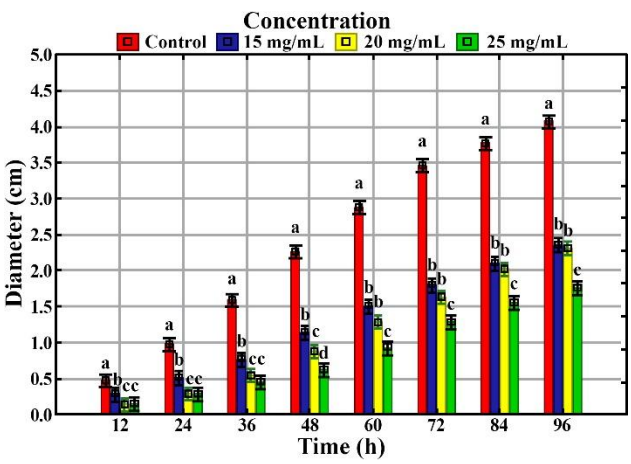

Fig. 5. Antifungal effect of the WPM extract over 96 h, with different concentrations of WPM extract. The same letters mean that the differences are not statistically significant, based on the results obtained with the Tukey test ($p<0.05$).

Conclusion

A comparative study of the antioxidant effect between different parts of *N. granadensis* was performed for the first time, and we have detected less antioxidant effect of the fruit extracts according to the significantly higher IC_{50} values observed in comparison with the extracts obtained from the whole plant. The methanolic extract obtained from the fruits in a Soxhlet extractor in relation to the methanolic extract obtained from the fruits using a maceration procedure revealed higher amounts of TPC when extracting in a Soxhlet extractor and also a higher antioxidant effect when using a Soxhlet extractor, based on a comparison between IC_{50}, GAE and TE values. When comparing IC_{50}, GAE and TE values of the extractions performed with methanol and with water in a Soxhlet extractor from the whole plant, a higher antioxidant effect was observed when the extraction was performed with methanol. The phenolic compounds detected by means of HPLC-MS were the same in the extracts obtained with methanol from fruits and from the whole plant, using a Soxhlet extractor, with the exception of a coumaric acid derivate present only in the whole plant extract. With regard to the observed antifungal effect of the WPM extract against *R. solani*, the possibility of a future antifungal treatment which is more environmentally friendly and has fewer side effects on human health when compared with synthetic fungicides should be considered.

Acknowledgement

This research was supported by the Vicerrectoría de Investigación y Desarrollo (Project Nr. 214.418.007-1.0) of the Universidad de Concepción. The authors are grateful for the technical support of Mrs. Claudia Flores.

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