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Antioxidative Properties from *Pitavia punctata* (Ruiz & Pav.) Mol. Leaves Infusion.

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ABSTRACT

Pitavia punctata (Ruiz & Pav.) Mol. is the only representative of Rutaceae in continental Chile and is an endangered species due to habitat destruction and other factors. Total content of phenols and flavonoids and antioxidant capacity were quantified from *P. punctata* leaves infusion. For its part, the infusion of the dry leaves has high amount of phenols and flavonoids (14.5 mg GAE/100mL and 7.8 mg QE/100mL of infusion, respectively) with antioxidant properties as shown in FRAP assay (89.6 Fe^{+2}/100mL of infusion), with an IC_{50} of 200 mg/100mL as showed in the DPPH assay.

Keywords: *Pitavia punctata*, antioxidants, FRAP, DPPH.

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INTRODUCTION

Pitavia punctata (R. et P.) Molina, belongs to a monotypic genus from Rutaceae, is an endemic arboreal species from Chilean Coast Range in Central Chile (35°21'S – 37°41'S, Figure 1) [1], which grows between the Maule river and the north of the Araucanía Region [2]. Its common names are "*pitao*", "*canelillo*" and "*pitrán*" [3-4]. Most of Rutaceae species grow in tropical forests and another group in template forests. Ruta and Citrus genus (a medicinal species and the citric group) belong to this taxon. Traditionally, it has been recognized that this family has near 900 species grouped in around 150 genres. The bigger diversity of this family is in Southern Africa and some places in Australia [5]. There are records about three traditional uses for *Pitavia punctata*: a) for the Araucanian people, the species is a medicinal resource, due to leaves infusions have anthelmintic properties [3]; b) it has been documented that near to Malleco province, people use *Pitavia punctata* wood for the elaboration of household utensils [6] and; c) in the context of various factors to explain the decrease of *Pitavia punctata* populations, it has been documented than near Concepción, the species has been used to get firewood [7]. Also, a large amount of polyphenols and antioxidant activity in species of Rutaceae have been described, e.g. citrus fruits [8] examples of species that belong to the Rutaceae family and have a high polyphenol content with antioxidant properties are *Citrus limon* (L.) Burm f. [9], *Zanthoxylum zanthoxyloides* (Lam.) Zepen & Timler (*Fagara zanthoxyloides Lam.)* [10] and *Vepris glaberrima* (Engl.) J.B. Hall (*Oriciopsis glaberrima* Engl.) [11].

MATERIALS AND METHODS

Plant Material and Extraction

Samples were taken in the Cauquenes province, in Maule region, (35°51'44"S – 72°25'53"O). Next to Cauquenes-Pelluhue M-50 route there are a *Nothofagus glauca* forest, with southwest exposure and an elevation of 407 meters above sea level. The collect date was July 25th, 2013. Samples were taxonomically determined by Luis Soto Cerda and stored at University of Concepción herbarium (Voucher N° 176999). All samples were dried at room temperature (20 – 30 °C) for three weeks in dark conditions.

Infusion preparation

For the infusion preparation, 0.5 g of dry leaves from *Pitavia punctata* were put into 200 mL of boiling water (equivalent to one teacup). After 10 min, infusion was filtered through Whatman No. 4 paper.

Extraction and quantification of organic acids exudates

A sample of dry leaves (1.75 mg) were washed with sterile water and transferred to a 10 mL flask containing 1.0 mL of Calcium chloride ($CaCl_2$, 0.5 mM), with aeration permanently by filtered air. Exudation process was realized in a period of 4 h at room temperature, and the leaves exudates were measured and the citrate was quantified by enzymatic methods.

Citric acid quantitative UV determination

The citrate quantification was carried out by the Cycling Enzymatic Kinetic Method (BEN, Biochemical Enterprise), by which the citric acid is changed in oxalacetate and acetate by the citrate lyase (CL). This reaction is helped by a secondary one, that transforms the oxalacetate originated before (and its decarboxilated product, pyruvate) in the presence of LDH, malate dehydrogenase (MDH) and NADH. Briefly, 1 mL of working reagent (LDH 500 U/L, NADH 0.1 mM, MDH 200 U/L and Good buffer 10 mM) were mixed with 25 µL of distilled water (blank reagent) or sample and were incubated for about 3 min at 25 ºC, then the absorbance was measured and 25 µL of working starter (CL 300 U/L in Good buffer 10 mM) were added. After 5 min at 25 ºC a second measured of the absorbance was made. The concentration of citric acid was calculated as follow:

Citric Acid (mg/mL): V/v x 1/Ɛd x MW/1000 x ΔAS

Where, V: total test volume (1.050 mL); v: sample volume (0.025 mL); d: pathlength (1 cm); Ɛ: molar coeff. NADH (6.3 L / mmol x cm); MW: citric acid MW (192.1) and ΔAS is the difference between the two absorbance measurements.

Total phenolic content (TPC)

The TPC was determined by the Folin–Ciocalteu method by Swain and Hillis, 1959 [12] used as standard gallic acid. The absorbance was measured at 750 nm using a spectrophotometer (Thermo Spectronic Genesys 10 UV). The results were expressed as GAE, (gallic acid equivalents) in mg GAE/100mL of infusion. Additional dilution was done if the absorbance value measured was over the linear range of the standard curve. All determinations were carried out in triplicate.

Total Flavonoid Content (TFC)

Total flavonoids content was determined using the method described previously [13]. Then, 200 µL of infusion or standard in methanol, the absorbance was read at 510 nm using a spectrophotometer. All determinations were carried out in triplicate. The results were expressed as quercetin equivalents (QE) in milligrams per 100 mL of infusion.

DPPH Decoloration Assay

The method was adapted to microwells used DPPH (1,1-diphenyl-2-picrilhydrazyl) assay according to the method of Brand-Williams et al. (1995) [14]. The absorbance was measured at 515 nm using a microplate reader spectrophotometer. Decoloration was calculated as percentage scavenging DPPH free radical:

% scavenging DPPH free radical = 100 × (1-AE/AD)

Where AE: solution absorbance after adding the infusion. The half-maximal inhibitory concentration (IC_{50}, mg/100mL) was calculated by linear regression analysis of three determinations.

FRAP (ferric reducing antioxidant power) assay

The FRAP assay was done according to Benzie and Strain, 1996 [15], using FRAP reagent (25 mL of 300 mM of acetate buffer, pH 3.6, 2.5 mL of 2,4,6-Tris (2-pyridyl)-s-triazine dissolved in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3$ * 6 H_2O). Then, 0.05 mL of the infusion or standard were added ($FeSO_4$ * $7H_2O$, Fe^{+2} concentration versus absorbance). The product (ferrous tripyridyltriazine complex) was read at a wavelength of 593 nm, using a spectrophotometer. The percentage of Fe^{+3} scavenging (reduction to Fe^{+2}) by comparison with the standard curve (µmol Fe^{+2} per 100mL of infusion).

RESULTS AND DISCUSSION

Citric Acid Content

The preparation of infusion protocol is based in other studies showing prepared for 10 min in boiling water (extraction is near to 94% of water-soluble phenolics) [16]. The infusion obtained from *Pitavia punctata* with this methodology showed great antioxidant capacity, for this reason the amount of citric acid in the leaves was determined, as it has been proposed that citrate have benefits for health like antioxidant. Thus, the presence of antioxidants like organic acids (e.g. citrate), may also contribute to the overall antioxidative observed effect [17]. The citric acid is very important for plants metabolism, due to the presence of this organic acid is necessary for several biochemical pathways, including formation of precursors for the aminoacids biosynthesis [18]. The results of the quantification of citric acid in leaves exudation leaves was 0.09 ± 0.001 mg/mg of dry leaf, equivalent at 9% of dry weight; this percentage is similar at that found in the infusion and provide antioxidant properties to the infusion.

Total Phenolic and Flavonoid Content

The presence of organic acids and polyphenols has shown multiple *in vitro* and *in vivo* biological properties, including antioxidant activities, among the phenolic antioxidants, the flavonoid family is the most important class [19]. Phenols and flavonoids concentration obtained from infusion were 14.5 ± 0.01 mg GAE/100mL and 7.8 ± 0.3 mg QE/100mL, respectively (Table 1).

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Table 1. Total phenolic, flavonoid contents and antioxidant activity of infusion obtained from *P. punctata* leaves.

Total Phenols mg GAE/100mL of infusion	Total Flavonoids mg Qe/100mL of infusion	FRAP µmol Fe^{+2}/100mL of infusion	DPPH (IC_{50}) mg/100mL of infusion
14.5 ± 0.01	7.8 ± 0.3	89.6 ± 0.5	200.0 ± 2.0

DPPH: 1,1-diphenyl-2-picrilhydrazyl; FRAP: ferric reducing antioxidant power.

Antioxidant Capacity

The DPPH assay shows a high antioxidant capacity (Table1) with an IC_{50} of 200 ± 2.0 mg/100mL of infusion, and the FRAP assay showed a ferric reducing antioxidant of 89.6 ± 0.5 µmol Fe^{+2}/100mL of infusion, confirming the good antioxidant capacity, which can be possible by the presence of high power antioxidant phenols as quercitine (member of the flavonoids family) [20] that has been reported in *Pitavia punctata* [21,22]. On the other hand, a significant correlation between DPPH and FRAP assays with the total phenolic content determined by the Folin-Ciocalteu method exists, indicating that *Pitavia punctata* leaves infusion has antioxidant capacities, due to the presence of phenolic compounds in the infusion, that contributes significantly to its antioxidant potential. This is mainly explained due to the presence of phenolic OH groups and π-electron conjugation to free radicals, which are scavenged and inactivated [23]. Silva et al,.1971 reported for *Pitavia punctata* the existence of polyphenols and diterpenes such as: β-sitosterol, daucosterin, quercetine, avicularin and 3-rhamnosylarabinoside [21] and Castro et al,. 2018 reported the existence of compound such as (-)-epichatechin, (-)-epichatechin-3-O-gallate, kaempferol and caffeic acid [22].

CONCLUSION

Pitavia punctata is an important natural resource of the swampy forests in Chilean Coast Range, because is an endemic and relict species with ancestral uses by original communities. In addition, this species is the only one Rutaceae from continental Chile.

There is necessary to develop research initiatives to discover species properties and executing native forests conservation strategies, including education activities. Increasing knowledge about ancestral uses and modern applications of native species is an important step to link natural resources with society priorities.

On the other hand, due to the ancestral history of Chilean forests, generate knowledge about evolutionary relationships between "different" species group are relevant to our planet dynamic understanding the role of free radicals the infusion of *Pitavia punctata* is an important antioxidant and can be used as therapeutic potential.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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