Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
23(1): 44-50, Jan./Feb. 2013

Variation in phenolic compounds of *Ugni molinae* populations and their potential use as antioxidant supplement

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Article

Received 20 Mar 2012
Accepted 11 Jul 2012
Available online 11 Oct 2012

Keywords:
plasma antioxidants
polyphenols
Ugni molinae populations

ISSN 0102-695X
DOI: 10.1590/S0102-695X2012005000122

Abstract: In the present work we carried out a comparative study of total phenolic contents and antioxidant capacity of aqueous leaf extracts of *Ugni molinae* Turcz., Myrtaceae (infusion and Soxhlet extracted) prepared from continent and Juan Fernández Island samples. The results revealed that total phenol content (TPC), tannins (TTC) and flavonoids (TFC) for *U. molinae* extracts (infusion and Soxhlet extracts) from island leaves were 38.5, 56.7 and 37.5% higher than those obtained with leaves from the continent, respectively. Also, HPLC profiles showed important differences between *U. molinae* populations. *In vitro* antioxidant capacity (scavenging of DPPH radical) for 1% infusion and aqueous extract (Soxhlet method) of *U. molinae* from island samples, was 15% greater than from continent samples. Further, *in vivo* impact of *U. molinae* intake (1% infusion) was studied in plasma samples obtained from healthy volunteers. Participants that consumed tea prepared with leaves from island population showed higher TBARS reduction and plasma antioxidant capacity (TEAC-CUPRAC) than those who consumed tea prepared with leaves from continental population. The conditions of the territory in which *U. molinae* populations growth could explain the differences in their composition and activity. According to results, island *U. molinae* populations could be an important source of study for the development of an antioxidant supplement, and thereby contribute to the use of this species that has becoming an ecological problem in the island.

Introduction

Ugni molinae Turcz. (Bull. Soc. Imp. Naturalistes Moscou 21: 579. 1848), Myrtaceae, is a native Chilean plant commonly known as *murtilla, murta or Chilean guava*. It is distributed from the Maule Region to Chiloé Island, including the Juan Fernández archipelago (Hoffmann, 1991). The leaves of this plant have been infused and used as an astringent for treating diarrhea and dysentery by the continent indigenous people. Several authors have determined the chemical composition and biological activity of continental *U. molinae* leaves (Avello, 2000; 2004; Avello & Pastene, 2005; Aguirre et al., 2006; Rubilar et al., 2006; Suwalsky et al., 2006; 2007; Avello et al., 2009; Rubilar et al., 2011). The presence of phenol-type substances such as catechin, epicatechin, myricetin, quercetin, kaempferol, and some of their glycosylated derivatives have also been described (Avello & Pastene, 2005; Rubilar et al., 2006; 2011). Moreover, studies intended to verify the benefits of the consumption of this plant for human health have shown that polar extracts of continental *U. molinae* stabilize free radicals generated in different systems (Avello, 2000; 2004). In our previous work, we established that plasma antioxidant capacity was increased significantly after an acute ingest of infusions prepared with continental *U. molinae* leaves sampled from a single location (VIII Region, Bío-Bío), according to traditional medicine practice (Avello & Pastene, 2005). The Juan Fernández archipelago is a national park located 670 km from the continent. It was declared a Biosphere Reserve by UNESCO. *U. molinae* was introduced to the archipelago (possibly by birds) from the continent. *U. molinae* behave like invasive species, occupying and clearly expanding into the habitat of the island's native species, competing heavily with and displacing the endemic species (Skottsberg, 1953; Ricci, 1989; Stuessy et al., 1998; Greimler et al., 2002; Cuevas et al., 2004). An option for controlling invasive species it might be their requirement for the food and pharmaceutical industry or for biotechnology development. Geo climatic factors are known to alter

the synthesis of secondary metabolites. Relevant climate factors include solar radiation, temperature, water, and soil (Davies & Schwinn, 2006). Therefore, the conditions of the territory in which populations of *U. molinae* develop could affect their chemical composition. Given the abovementioned, quantitative differences in total phenolic contents and bio-activities are expected to occur between populations of *U. molinae*. Hence, the first aim of this work was to compare total phenolic contents of infusions and aqueous extracts prepared with *Ugni* leaves from the continent and Juan Fernández Island. In second place, we plan to evaluate the impact of both *U. molinae* populations on the plasma antioxidant capacity of healthy volunteers before and after they drink infusions prepared in the way they normally ingest their herbal native infusions.

Materials and Methods

Vegetable matter

The biological material was collected from Bío-Bío Region, (36º00' and 38º30' S) and from Juan Fernández archipelago (Robinson Crusoe Island, 33º36' and 33º46' S), Chile. The leaves of the plants were collected in november-december 2008, when the plant was flowering. The species was identified by the taxonomist, Dr. Roberto Rodríguez, of the Department of Botany, Faculty of Natural and Oceanographic Sciences, Universidad de Concepción (CONC 146511 y 116887, respectively).

Obtaining aqueous extracts

Dried leaves from both *Ugni molinae* Turcz., Myrtaceae, populations were washed, air-dried, and ground to a fine powder. Powdered *U. molinae* leaves (50 g) were extracted in a Soxhlet apparatus with water until exhausting the vegetable matter. The mass: solvent ratio was 1:6. The extracts obtained from the samples of both populations were freeze-dried and stored in a dry place, protected from the light, until their use.

Obtaining infusions

Infusions were prepared with 1 g of dried, ground leaves from each population. For this, 100 mL of water (100 °C) were added to the leaves, which were left to steep for 5 min. The samples to be used for qualitative and quantitative analyses were freeze-dried.

Total polyphenol contents

Total polyphenol contents (TPC) were determined spectrophotometrically according to Velioglu et al. (1998), using the Folin-Ciocalteu reagent (Sigma, MO, USA). Briefly, aliquots (0.5 mL) of test samples were mixed with 25 mL water, 2.5 mL Folin-Ciocalteu reagent (Merck, Germany), and 10 mL 20% Na_2CO_3, and then completed to 50 mL with water. The mixtures were shaken for 30 min, then allowed to stand for 30 min. Absorbance was registered at 765 nm using gallic acid as a standard. Total tannin (TTC) and flavonoid (TFC) contents were determined, respectively, according to Lastra et al. (2000) and Salamanca et al. (2007).

HPLC analysis

Extracts (3 mg/mL) were separated by RP-HPLC using a Lachrom instrument, equipped with a 250 × 4.6 mm, 5 µm, Kromasil KR100-5C18 column (Eka Chemicals AB, Bohus, Sweden). A gradient elution was performed by varying the proportion of solvent A (double distilled water containing 0.1% TFA, v/v) to solvent B (acetonitrile containing 0.1% TFA) with a flow of 1 mL/min. The following gradient was used: 0-25 min, 10-25% of B in A; 25-30 min, 25-75% of B in A and then bring mobile phase composition back to the initial condition in 5 min to the next run. For flavonoids, detection was at 350 nm using a diode array detector. For hydrolysable tannins (gallic acid derivates) and catechins, detection was done at 280 nm. When reference substances were available some compounds were grouped and tentatively identified by matching their retention times (t_R) and online UV spectra. Although the other compounds (particularly some rare myricetin and quercetin-3-*O*-glycosides) could not be wholly identified, they were characterized according to their class on the basis of their UV-VIS spectra and data reported previously in literature (Rubilar et al., 2006). The reagents used for analysis were all HPLC grade (Merck, Germany). Peak assignment was done by comparing the tR with those of pure standard substances, all purchased from Sigma (gallic acid, catechin, epicatechin, isoquercitrin, isorhamnetin, quercetin, myricetin, myricitrin, kaempferol and ellagic acid).

Antioxidant capacity in vitro*: stabilization of the 1, 1-diphenil-2-picryl-hydrazyl (DPPH) radical*

The DPPH radical was stabilized as described by Joyeux et al. (1995) for both extracts and the infusions made from the two *U. molinae* populations. The results were expressed as a percentage of the discoloration of the radical. Gallic acid (Merck, Germany) was used as a standard.

Administration of infusions

The infusions made with the two *U. molinae* populations were administered twice daily (11 am and 17 pm) for three days, simulating the dosage used in popular medicine (1%). This study was carried out with the approval of the Ethics Committee of University of Concepción (NºVRID 290/2012), and the volunteers were recruited with informed consent, and following guidelines of the Declaration of Helsinki and Tokyo for humans.

Plasma preparation

Blood was obtained from healthy volunteers (n=24, 20-30 years of age, non-smokers, normal range of body mass index, normolipemic, non-diabetic, following a normal diet) through venipuncture before and after drinking the teas (1%) made with leaves from continental (n=12) and island (n=12) populations of *U. molinae*. EDTA (2.7 mM) was used as an anticoagulant in the samples. The plasma was separated after centrifuging at 800 x g at 4 ºC for 15 min according to Avello & Pastene (2005).

Conjugated dienes

The formation of conjugated dienes was determined by UV absorption at 234 nm (Esterbauer et al., 1989) using a spectrophotometer (Shimadzu UV-VIS 1601).

Thiobarbituric acid reactive substances (TBARS)

Plasma samples (500 µL) were mixed with 1 mL of the TBARS reagent and the mixture was incubated at 100 °C for 30 min. After cooling on ice and centrifuging at 1800 *g* for 15 min (Kubota, Japan), the absorbance of the supernatants was measured at 532 nm (Jasco, Japan). The results were expressed in terms of percentage of protection (Gugliucci, 1996).

Trolox equivalent antioxidant capacity-Cupric ion reducing antioxidant capacity (TEAC-CUPRAC assay).

TEAC-CUPRAC indexes were determined according to Apak et al. (2007). For this, a $CuCl_2$ solution (1.0×10^{-2} M) was prepared by dissolving 0.4262 g $CuCl_2 \times 2H_2O$ in water and diluting to 250 mL. Ammonium acetate buffer (1.0 M, pH 7.0) was prepared by dissolving 19.27 g NH_4Ac in water and diluting to 250 mL. Neocuproine (Nc) solution (7.5×10^{-3} M) was prepared daily by dissolving 0.039 g Nc in 96% ethanol and diluting to 25 mL with ethanol. Trolox (1.0×10^{-3} M) was prepared in 96% ethanol. The assay was performed by adding 1 mL 10^{-2} M Cu^{2+}+1 mL 7.5×10^{-3} M neocuproine+1 mL 1M NH_4Ac. The antioxidant sample (or standard) solution (x mL) and H_2O (1-X mL) were added to the initial mixture in order to make a final volume of 4.1 mL. The final absorbance was measured at 450 nm.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. Differences were considered significant at $p<0.01$.

Results and discussion

The total phenol contents of the aqueous extracts (Soxhlet method) differed significantly. So, the island population of *Ugni molinae* Turcz., Myrtaceae, contained 38.5% more total phenols (TPC) than continental population (Figure 1A). Likewise, compared with the continental population, aqueous extracts from the island population showed 56.7% and 37.5% more total tannins (TTC) and flavonoids (TFC), respectively (Figure 1B). *In vitro* antioxidant capacity (scavenging of DPPH radical) for 1% infusion and aqueous extract (Soxhlet method) of *U. molinae* from island samples, was 15% greater than from continent samples (Figure 1C). These differences were clearly illustrated in HPLC profiles depicted in Figures 2A and B. In the extracts from the continental population, HPLC analysis suggested the presence of phenolic acids, flavan-3-ols and flavonols derivates (Figure 2A). However, its identity could not be assigned in all cases because certain gallotannins and myricetin and quercetin derivates currently were not available in our laboratory. Nevertheless, it was possible to tentatively assign the identity of gallic acid (t_R=4.7 min), catechin (t_R=9.8), epicatechin (t_R=12.5), myricetin 3-*O*-rhamnoside (myricitrin; t_R=19.2), quercetin-3-*O*-glucoside (isoquercitrin; t_R=22.0) and ellagic acid (t_R=31.8). In the aqueous extract of the island population, we could clearly identify some of the polyphenols described previously by Rubilar et al. (2006; 2011) such as gallic acid derivates (hydrolysable tannins), flavan-3-ols (epicatechin) and glycosides of myricetin, quercetin (Figure 2B). These substances are widely known for their antioxidant, antinociceptive, antiallergic and antitumoral activities (Yokoshimo & Moriwaki, 2005; Meotti et al., 2006; Shimosaki et al., 2011). Altogether, HPLC registers suggest that insular population have similar flavonoids and tannins than continental population but its levels, particularly flavonoids, are highest. These results are in accordance with the observed difference in the total

phenolic, tannins and flavonoids contents (Figure 1A). It should be noted that the study carried out by Rubilar et al. (2006) was done using a continental population collected in the Araucanía Region (IX). In the present work, our continental sample was extracted in Bío-Bío region (VIII). In both cases, there is more than 800 km between insular and continental sampling zones. Geologically, Juan Fernández is an archipelago of volcanic origins, and it has a warm, temperate, subtropical, marine climate with high environmental humidity. This climate creates special conditions in the sector and allows the development of exuberant, unique vegetation. In turn, the Bío-Bío Region is a transition zone between the temperate climates of central Chile and the rainy climates found southern of the "*rio Laja*". The predominant climate in this region is temperate Mediterranean and, in general, the soils are classified as miscellaneous (www.meteochile.cl). The high humidity and volcanic soils of the Juan Fernández archipelago could influence the biosynthesis of phenolic compounds. Correlations have been reported between humidity conditions of a territory and the levels of phenolic compounds. On the other hand, a highly fertile environment with elevated iron hydroxide and aluminum hydroxide contents (Ritter et al., 2003). Although contributions of nitrogen, phosphorous, potassium, and sulfur are described as important, their deficiency in volcanic soils could stimulate the biosynthesis of phenolic compounds. These mechanisms could be associated with the activity of key enzymes in phenolic biosynthesis such as phenylalanine ammonia-lyase (PAL) (Davies & Schwinn, 2006). The mechanisms used by plants to adapt to stressful situations (*e.g.*, an introduced species in an unknown environment) are also important; the resulting defense mechanisms have a high participation of phenolic compounds, as do the allelopathic mechanisms used to protect against pathogens and predators (Waniska, 2000; Vivanco et al., 2005; Vermerris & Nicholson, 2006).

In order to evaluate if the acute oral intake of teas prepared from two populations of *U. molinae* could modify antioxidant status in humans we design a pilot-scale study. In this trial, plasma antioxidant capacity of 24 healthy volunteers was measured before and after the administration of infusions (1%) of *U. molinae* leaves from the continent and island through the formation of conjugated dienes, TBARS, and TEAC-CUPRAC. None of the volunteers reported adverse effects. TBARS formation was reduced in a 31.22% for those participants who drank tea from the island population, as opposed to 19.33% for the group that drank the tea made using the continental population (Figure 3A). Although our measurements of conjugated dienes did not reveal a general overview of the influence of the assayed infusions on plasma antioxidant protection at this level, we did note a slight protection

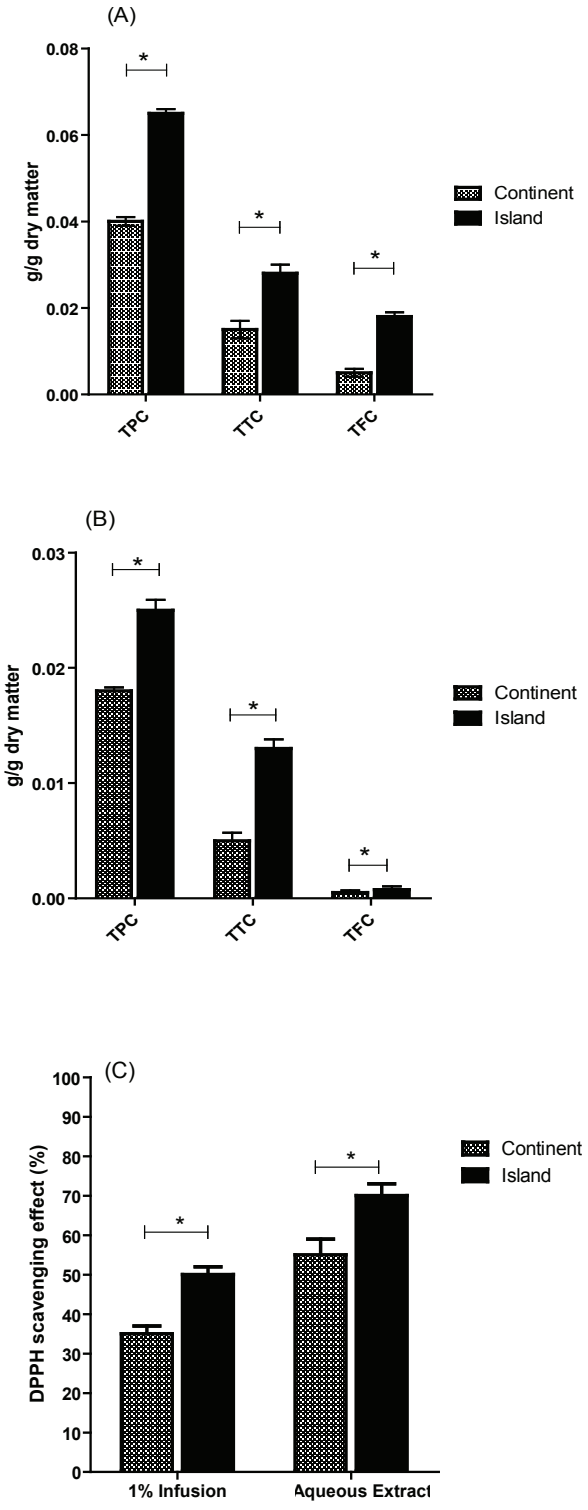

Figure 1. Total polyphenol contents (TPC), Total tannin contents (TTC), and Total flavonoid contents (TFC) of aqueous extracts (A) and infusions (B) from island and continental populations of *Ugni molinae.* (C) DPPH scavenging capacity of 1 % infusions and aqueous extracts from island and continental populations of *U. molinae*. Values are the mean±SD; n=3.*p<0.01.

(2.66%) associated with the ingestion of the teas made from the island population (Figure 3A). Fluctuations in the plasma Trolox equivalents (TEAC-CUPRAC), could be ascribed both to an increased ingest of antioxidants (from natural or synthetic sources) or to pathological states that promote depletion of endogenous antioxidants (Apak et al., 2007). The results of our study reveal that TEAC-CUPRAC indexes were improved after oral intake of U. molinae teas from both populations. Interestingly, plasma samples of participants who consumed tea prepared with island plants has 316.8 µM more Trolox equivalents than those samples analyzed in the continent group (p<0,05) (Figure 3B). Such results suggest that positive correlation between polyphenol contents and the *in vivo* antioxidant activity might be associated to *U. molinae* intake. The phenolic compounds described for this species predominantly are flavonoid glycosides and high molecular weight tannins whose bioavailability remains uncertain. However, important evidence suggests that these compounds could be substrate for intestinal microbiota (Selma et al., 2009). Therefore, unbound polyphenols (genins) or even new molecules formed in gastrointestinal tract might be responsible of the acute increments in the antioxidant status of human plasma after intake of polyphenol-rich beverages and foods (Yokoshimo & Moriwaki, 2005; Landete, 2011). These associations are merely speculative and need to be duly clarified trough pharmacokinetic studies. Encouraged for the results of this work, we plan to lead our efforts to the study of chemical profiles for phenolic compounds and its metabolites in the blood of humans after *U. moline* tea intake.

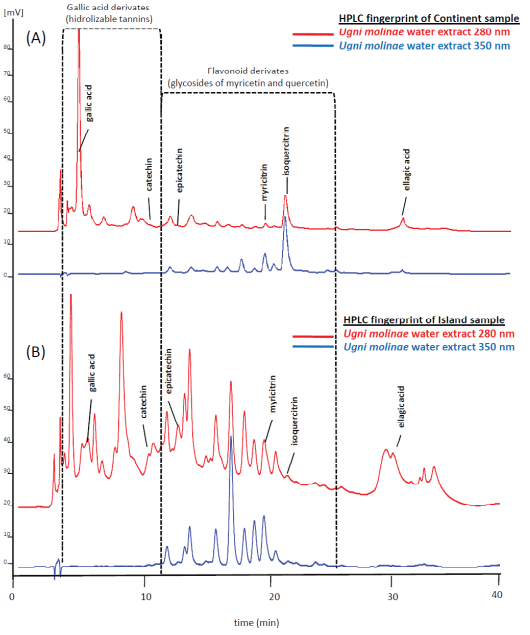

Figure 2. HPLC analysis of *Ugni molinae* aqueous extracts. A. Chromatogram of the aqueous extract of continental *U. molinae*; B. Chromatogram of the aqueous extract of island *U. molinae.*

Conclusions

Our results reveal that total phenolic contents and *in vitro* antioxidant capacity of infusions and

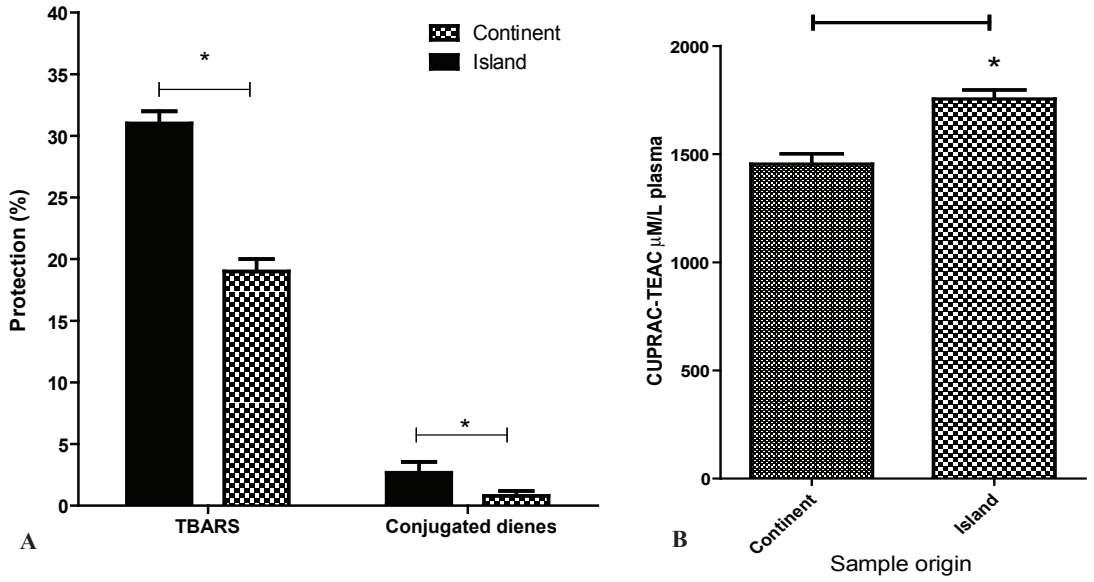

Figure 3. A. Protection (expressed as a percentage) following the ingestion of infusions made using island and continental populations of *Ugni molinae* in the formation of TBARS and Conjugated Dienes. *B. Trolox* equivalents (TEAC) following the ingestion of infusions of island and continental populations of U. molinae through TEAC-CUPRAC Values are the mean±SD; n=12, by group. *p<0.01.

aqueous extracts prepared from *Ugni* leaves of Juan Fernández archipelago are higher than those of the continent. Conditions of the territory in which the *Ugni molinae* Turcz., Myrtaceae, populations growth may explain such differences. The plasma antioxidant capacity of the healthy volunteers differed before and after the ingestion of teas made from the leaves of the continental and island populations, following the dosages of traditional medicine, favoring the island populations. These results let us to propose the population of the island as a source of study for the development of an antioxidant supplement, and thereby contribute to the use of this species that has becoming an ecological problem in the island.

Acknowledgments

This work was supported by CONICYT (24091006) and the postgraduate council of the University of Concepción, (D.I. 210.074.043), and Proyecto Anillo (ADI-38), Anillo ACT-38, Proyecto Nuestra Flora de Chile, CONAF, Armada de Chile.

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