Chilean berry *Ugni molinae* Turcz fruit and leaves: interesting source of antioxidant, antimicrobial and anti-tyrosinase compounds

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ABSTRACT

The knowledge of the biological properties of fruits and leaves of murta (*Ugni molinae* Turcz) has been owned by native Chilean culture. The present study investigated the phenolic content, the antioxidant, antimicrobial and anti-tyrosinase activities of different murta fruit and leaves extracts to approach their uses on future food, pharmaceutical and cosmetic applications. Extractions of murta fruit and leaves were carried out under water, ethanol and ethanol 50%. Phenolic content of these extracts was measured through Folin Ciocalteu test and the antioxidant power by four different antioxidant systems (ORAC, FRAP, DPPH and TEAC assays) owing to elucidate the main mechanism of antioxidant action depending on the chemical composition of the extracts. Some flavonoids, such as rutin, isoquercitrin and quercitrin hydrate were identified and quantified through HPLC analysis. Antimicrobial activity was determined measuring minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values against *Escherichia coli* and *Listeria monocytogenes,* and the effect of these extracts on *L. monocytogenes* was confirmed by flow cytometry. Results indicated phenolic content and chemical composition of murta fruit and leaf extracts were considerably higher than other fruits and plants, showing promising perspectives as potential ingredients for the cosmetic and food industries. Murta leaves presented higher antioxidant, antimicrobial and anti-tyrosinase properties if compared with murta fruit, principally the hydroalcoholic extract.

Keywords: murta, natural extract, polyphenols, antioxidant, antimicrobial, tyrosinase inhibitor

Chemical compounds studied in this article: 2,2-diphenyl-1-pícrylhydrazyl (DPPH) (PubChem CID 74358); 2,2´-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) (PubChem CID 90658258); gallic acid (PubChem CID 370); Trolox ((PubChem CID 40634); sodium carbonate (PubChem CID 10340), AAPH (2,2-Azobis (2-methylpropionamidine) dihydrochloride (PubChem CID 76344); ferric 2,4,6-tripyridyl-s-triazine (TPTZ) (PubChem CID 77258); fluorescein (PubChem CID 16850); arbutin (PubChem CID 440936); kojic acid (PubChem CID 3840).

1. Introduction

During last years, there is an increasing interest in plant-derived products due to an increasing demand for: i) natural food additives/products by consumers; ii) chemical diversity in screening programs; and iii) seeking pharmacological drugs from natural products without toxicological effects. Scientific community studies have focused on natural products from medicinal plants, either as pure compounds or as plant extracts, as an alternative solution for these demands due to their chemical composition rich in bioactive compounds (Rios & Recio, 2005, Sasidharan et al., 2011).

Composition and traditional folk medicine of berries suggest significant health benefits, but few studies to date have investigated these potentials through chemical and biological activities (Fredes, 2009; Junqueira et al., 2015; Schreckinger, Lotton, Lila, & Gonzalez de Mejia, 2010). It has been also confirmed that berries have higher antioxidant capacity than other fruits and vegetables due to their high content in polyphenols. Several studies have also found the consumption of berries is effective in prevention and treatment of some diseases (Erlund et al., 2008; Suwalsky et al., 2007). Murta, murtilla, or scientifically called *Ugni molinae* Turcz, is an aromatic Myrthacea specie native growing in the coast and pre-Andean mountains of South Chile. This wild perennial shrub is widely used in folk medicine by indigenous people, including Mapuche, Puelche and Pehuenche cultures, for the treatment of several ailments, such as diarrhea, ulcers, fever, inflammations and migraines. The scientific interest by this native shrub has emerged recently, and since then, the chemical composition of this plant, both fruit and leaves, and new potential applications of their compounds have been studied (Hauser et al., 2014; Jofré et al., 2016; Junqueira et al., 2015; Peña-Cerda., et al., 2017; Rubilar et al., 2011; Suwalsky et al., 2007). In this work, different extractions of fruit and leaves were

performed in order to obtain the highest amount of bioactive compounds with antioxidant, antimicrobial and anti-tyrosinase activities. The aim of this study was to present a relation between these activities with their chemical composition depending their polarity and affinity with extractive solvents. Antioxidant activities were measured through different assays owing to understand mechanisms of action of their active compounds. Antimicrobial activity of murta extracts have been previously studied by other authors (Hauser et al. 2014; Junqueira et al., 2015; Shene et al., 2009), but their MIC and MBC values against pathogen bacteria have never been reported. On the other hand, nowadays there is also an increasing interest by cosmetic industry for the searching of natural tyrosinase inhibitors in order to substitute synthetic compounds used on anti-pigmenting and skin protectors. Tyrosinase is the key enzyme involved in both mammalian melanogenesis and fruit or fungi enzymatic browning (Chang, 2009, Kim & Uyama, 2005). Anti-tyrosinase compounds may help combat wrinkles and other signs of premature aging, and delay some vegetable food products darkening. The principal aim of this work was not finding the complete list of compounds from murta fruit and leaves, but obtaining relevant data related to their antioxidant, antimicrobial and anti-tyrosinase activities.

Earlier studies were already focused on identifying the chemical composition of murta fruit and leaves extracts under different solvents (Junqueira et al., 2015; Jofré et al., 2016; López et al., 2017; Peña-Cerda., et al., 2017). Thus, the objective of this work was to analyze the antioxidant, antimicrobial and anti-tyrosinase activities of murta fruit and leaves owing to validate their properties and traditional uses through scientific data for a proper application on functional food, nutraceutical and cosmetic products.

2. Materials and Methods

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99 *2.1. Plant material and reagents*

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101 *Ugni Molinae* fruit and leaves were collected from Yumbel in the VIII Region of
102 Biobío in Chile by the company "Agrícola Pallauquen E.I.R.L. (37°50´S, 72°35´W). The
103 collected materials were dried (at 35 ºC) to a constant weight and ground in a coffee
104 grinder. 2,2´-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), Folin Ciocalteu
105 phenol reagent, 2,2-diphenyl-1-pícrylhydrazyl (DPPH), anhydrous sodium carbonate,
106 gallic acid, AAPH (2,2-Azobis (2-methylpropionamidine) dihydrochloride, ferric 2,4,6-
107 tripyridyl-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
108 (Trolox) were purchased from Sigma Aldrich (Santiago, Chile). Kojic acid, arbutin,
109 catechin hydrate, rutin, isoquercitrin, ellagic acid dihydrate and quercitrin hydrate were
110 purchased from Sigma Aldrich Quimica S.A. (Madrid, Spain).

111 Culture preparation: *Listeria monocytogenes* CECT 934 (ATCC 19114) and
112 *Escherichia coli* CECT 434 (ATCC 25922) were chosen due to their relevance in food-
113 borne illness. Strains were obtained from the Spanish Type Culture Collection (CECT,
114 Valencia, Spain), and stored in Tryptone soy broth (TSB) (Scharlab, Barcelona, Spain) at
115 -80 °C until needed. For experimental use, the stock cultures were maintained on
116 Tryptone soy agar (TSA) (Scharlab, Barcelona, Spain) slants at 4 °C and transferred
117 monthly. Prior to each experiment, a loopful of each strain was transferred to 10 mL of
118 TSB and incubated at 37 °C for 18 h to obtain fresh early-stationary phase cells.

119 Strain of *Penicillium expansum* number 2278 was supplied by CECT (Valencia,
120 Spain). *Penicillium expansum* was grown on PDA (Potato dextrose agar, Scharlab,
121 Barcelona, Spain) in petri dishes at 28 ºC for 7 days. Previous experiment, conidia were
122 collected by flooding the surface of the inoculated petri dishes with 0.05% of Tween 40

and peptone water scraping the mycelial surface with a spatula. 5 mL of this suspension were transferred to sterile plastic tubes and shaken to obtain a homogenous suspension of conidia. The conidial suspensions were diluted until to 10^6 spores/mL. The Neubauer (Counting Neubauer chamber, Marienfeld, Germany) method was used to count the spore concentration visualizing with a Neubauer camera in a professional biological optic microscope Motic B3 Series (Barcelona, Spain).

2.2. Extraction of active compounds from murta fruit and leaves

Each plant material (fruit and leaves) were extracted using absolute ethanol, ethanol 50% and distilled water using a solvent:solid ratio of 500:1 in order to study the effect of the solvent polarity on the extraction of the most relevant antioxidant compounds, and to elucidate its posterior application. The extractions were carried out at 40 °C during 3 h with an agitation of 150 rpm. Samples were centrifuged and the upper phases were collected and used to analyze the phenolic content and the antioxidant activities. Extracts obtained were named as (F-E1, F-E2, F-E3) and (L-E1, L-E2, L-E3) for extracts under ethanol (E1), ethanol 50% (E2) and water (E3) of murta fruit (F) and leaves (L), respectively. In the case of antimicrobial assays, extracts were concentrated until a final concentration of 0.2 g sample/mL extract, and they were named with (´), F-E1´ in the case of ethanolic murta fruit extract, as an example.

2.3. Study of murta fruit and leaves extracts composition

Hydroalcoholic murta fruit and leaves extracts were analyzed by high performance liquid chromatography HPLC-PDA-FL to identify their major flavonoid compounds

using a Waters 2695 Separation Module equipped with a SunFire C18 Column (3.5 µm, 3.0 x 150 mm) and a Waters 2996 Photodiode Array Detector, operating at 35 ºC at a flow rate of 0.5 mL/min. The injection volume was 20 µL. A nonlinear gradient of solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid) in the following proportions: 0 min, 85% A, 15% B; 1 min, 85% A, 15% B; 30 min, 40 % A, 60 % B; 40 min, 0% A, 100% B; 45 min, 0% A, 100% B; 47 min, 85% A, 15% B; 48 min 85 % A, 15 % B. Quantification of antioxidant compounds were based on external standard calibration methods. Integration and data elaboration were performed using Empower 3 software, Build 3471. The standard solutions of catechin hydrate, rutin, isoquercitrin, ellagic acid dihydrate, quercitrin hydrate and narcissin were prepared in 50% ethanol at a concentration range of 0,002 – 0,009 mg/mL.

Furthermore, due to the large composition of these extracts, a search for other compounds from data of previous and recent studies were included in order to end up with a more complete list of chemical compounds.

2.4. Determination of total phenolic content

Total phenolic content was estimated colorimetrically by means of the Folin–Ciocalteu method with slight modifications (Singleton, Orthofer, & Lamuela-Raventós, 1999). A volume of 100 µL of each extract was mixed with 3100 µL of distilled water and 200 µL of Folin-Ciocalteu reagent. The mixture was shaken and stored in the dark for 5 min. Subsequently, 600 µL of an anhydrous sodium carbonate solution (20% v/v) were added and stirred until homogeneous color was observed. After 2 h of reaction, the coloration of the samples was recorded as their absorbance at 765 nm. Results were expressed as equivalent mg gallic acid per g of dried sample.

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174 *2.5. Antioxidant activity studies of murta extracts*

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176 Because natural extracts are composed by several compounds with different
177 mechanisms of antioxidant action, the antioxidant power of murta fruit and leaves was
178 measured through four complementary antioxidant assays: oxygen radical absorption
179 capacity (ORAC), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-
180 picrylhydrazil (DPPH) and Trolox equivalent antioxidant capacity (TEAC). ORAC
181 measures the antioxidant activity through hydrogen atom transference, FRAP is a totally
182 electron transfer based method and TEAC and DPPH measure antioxidant activity as a
183 result of both mechanisms (Deng et al., 2013; Frankel & Meyer, 2000; Prior, Wu, &
184 Schaich, 2005). All antioxidant results were expressed as mg Trolox/g fruit or leaves.

185 ORAC-FL assay was carried out following Ou et al. method (2001) with some
186 modifications (Dávalos, Bartolomé, Suberviola, & Gómez-Cordovés, 2003; Ou,
187 Hampsch-Woodill, & Prior, 2001). The reaction was carried out in 75 mM phosphate
188 buffer (pH 7.4). The mixture of extract/sample (20 mL) and fluorescein (120 mL; 70 nM
189 final concentration) was preincubated for 15 min at 37 ºC. AAPH (2,2-Azobis (2-
190 methylpropionamidine) dihydrochloride) solution (60 mL; 12 mM final concentration)
191 was added rapidly using a multichannel pipette. The plate was immediately placed in the
192 reader and the fluorescence recorded every minute for 120 min (excitation wavelength
193 485 nm, emission wavelength 520 nm). A blank sample using phosphate buffer instead
194 of the antioxidant solution and eight calibration solutions using Trolox were also carried
195 out in each assay. Results were calculated based on the differences in areas under the
196 fluorescein decay curve between that of the blank and those of the samples.

FRAP assay is a totally electron transfer based method and measures reduction of ferric 2,4,6-tripyridyl-*s*-triazine (TPTZ) to a colored product, and it was carried out in accordance with Lu et al. (2011) studies (Lu et al., 2011). The FRAP reagent was prepared with 2.5 mL of a TPTZ solution (10 mM) in hydrochloric acid (40 mM) and 2.5 mL of a $FeCl_3$ solution (20 mM) mixed with 25 mL of an acetate buffer (0.3 M, pH 3.6). The reaction mixture (2850 µL FRAP reagent + 150 µL extract) was stored at room temperature for 30 min and the absorbance was measured at 593 nm.

DPPH method is based on the bleaching rate of the radical stable 2,2-diphenyl-1-picrylhydrazyl monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical compound its absorption decreases. TEAC assay is based on the inhibition of the cationic radical 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\cdot+}$), which has a characteristic long wavelength absorption maxima at 734 nm. Both radicals can be neutralized either by direct reduction via electron transfers or by radical quenching via H atom transfer (Prior et al., 2005). In both assays, the percentage inhibition values were calculated using this equation 1:

$$I\ (\%) = (A_{control} - A_{sample}) / A_{control} \times 100 \qquad (1)$$

DPPH radical-scavenging activity of murta extracts was evaluated according to the method described by Okada and Okada with some modifications (Lopez de Dicastillo et al., 2011; Okada & Okada, 1998). Extracts were incubated with DPPH solution for 30 min in the dark at room temperature, and absorbance was determined at 517 nm using a spectrophotometer. ABTS radical cations were produced by reacting 7 mM ABTS in water with 2.45 mM potassium persulfate ($K_2S_2O_8$) and then stored in darkness at room temperature for 16 h. $ABTS^{\cdot+}$ radical solution was diluted to give absorbance value of 1 at 734 nm. 3 mL of $ABTS^{\cdot+}$ radical solution were exposed to 300 µL of murta extracts

and absorbance were monitored through time. All experiments were performed in triplicate.

2.6. Antimicrobial activity studies

2.6.1. Determination of MIC and MBC values

The antimicrobial activity of murta fruit and leaf extracts against *L. monocytogenes* and *E. coli*, as Gram-positive and Gram-negative bacteria models, respectively. Serial dilutions of 0.2 g/mL concentrated extracts (E1´, E2´ and E3´) were made up in sterile buffer to study the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The antibacterial activities of the plant extracts were determined using sterile 96-well plates (Wiegand, Hilpert, & Hancock, 2008**).** Cell cultures of each microorganism in stationary phase measured at 600 nm were diluted in TSB and incubated at 37 ºC until reach the exponential phase corresponding with an optical density of 0.2 and a bacterial concentration of 10^6 CFU/mL. 20 µL of these solutions were added to the 200 µL final volume wells, obtaining a final concentration of 10^5 CFU/mL bacteria. Wells 1-9 received the plant extract, each extract E1´, E2´ and E3´ in triplicate, and wells 10-12 served as controls (ethanol, ethanol 50% and distilled water, respectively). Plant extracts were serially diluted from wells A-H to create a concentration sequence. The deep-wells were incubated for 24 h at 37 ºC. After the incubation period, 5 µL of every well was grown in a Petri dish. The lowest plant extract concentration that inhibited the pathogen microorganism was reported as the MIC. The MBC was the lowest concentration at which bacteria failed to grow in TSB and were not cultivable after platting onto TSA (Smith-Palmer, Stewart, & Fyfe, 1998).

2.6.2. Flow Cytometry

The viability of *L. monocytogenes* in contact with hydroalcoholic plant extracts (F-E'2 & L-E'2 for fruit and leaves extract, respectively) was studied by flow cytometry. Dilutions of these extracts in peptone water were added to 10 mL of TSB in order to reach a final concentration corresponding to the MIC value determined previously. Briefly, 100 µL of *L. monocytogenes* in exponential phase was inoculated in each test tube and incubated for 24 h at 37 °C. After the incubation period, the samples were centrifuged and washed twice in sterile saline solution (0.8% NaCl). The cells were stained with the cell-permeant double-stranded DNA fluorochrome SYTO-13 (Molecular Probes Europe BV, Netherlands) and with propidium iodide solution (PI, Calbiochem). SYTO-13 stains the nucleic acid in all cells, while PI stains the nucleic acid in cells with a damaged membrane. The studies were conducted in the Central Service for Experimental Research at the University of Valencia (Spain), performed with a BD FACSVerse™ (Becton Dickson, USA) Flow Cytometer. Each cell was characterized by four optical parameters: side scatter (SSC), forward scatter (FSC), green fluorescence for SYTO-13 (excitation light 497 nm and emission light 520 nm) and red fluorescence for PI (excitation light 493 nm and emission light 630 nm). The software BD FACS Suite v. 1.0.3.2924 (Becton Dickson, USA) was used to collect the data. An untreated control of the pathogen microorganism was also analyzed.

2.6.3. Antifungal activity

The antifungal activity of murta extracts was studied against *Penicillium expansum*. In this study, the hydroalcoholic extracts were selected due to their highest antimicrobial activity. For that, 10 µL of a conidial suspension of *Penicillium expansum* diluted until to 10^6 spores/mL were added in Potato Dextrose Broth (Scharlab, Barcelona, Spain) and

extracts were added at MBC concentrations. At the same time, tubes without antimicrobial agents were used as control. Samples were incubated at 28 ºC for 4 days. After the incubation period, samples were centrifuged and the obtained fungal pellets were weight and compared with the control. Three replicates of each sample were evaluated.

2.7. Determination of anti-tyrosinase activity

The colorimetric tyrosinase assay was performed using the method described by Momtaz et al. (2008) with minor modifications. Murta extracts with highest phenolic content were analyzed. The source of tyrosinase enzyme was *Agaricus bisporus*, an edible mushroom species that is native to grasslands in Europe and North America. The activity of tyrosinase inhibition was determined spectrophotometrically by monitoring dopachrome formation at 492 nm. The potential tyrosinase inhibitor will cause a decrease in dopachrome absorption. In a 96-well plate, 70 µL of each sample solution of different concentrations were combined with 30 µL of tyrosinase (333 units/mL in phosphate buffer, pH 6.5) in triplicate. After incubation at 25 ºC for 5 min, 110 µL of substrate (2mM L-DOPA) were added to each well. Kojic acid and arbutin were used as control drugs. The extracts and pure compounds were diluted in 50 mM potassium phosphate buffer (pH 6.5) to appropriate concentrations. Microliter plates were incubated for 30 min at 25 ºC. Optical densities of the wells were then determined at 492 nm with the SpectraMAx M2 Microplate Reader from Molecular Devices. The fifty percent inhibition concentration (IC_{50}) value was determined by the use of SoftMax Pro Software. The inhibitory effects of the test samples were expressed as the percentage of tyrosinase inhibition as follows:

$$\%\,\text{inhibition} = \left[\frac{(A-B)-(C-D)}{(A-B)}\right] \times 100 \quad (2)$$

where A and B denote the optical density at 492 nm of the mixture without test sample (L-DOPA mixed with enzyme in buffer; the control) and without test sample and enzyme (L-DOPA in buffer; the blank), respectively. C and D denote those with test sample and enzyme (L-DOPA mixed with enzyme and test sample in buffer; the reaction mixture) and without enzyme (L-DOPA mixed with test sample in buffer; the blank of C).

2.7. Statistical Analysis

One-way analyses of variance were carried out using the SPSS computer program (SPSS Inc., Chicago, IL). Differences in pairs of mean values were evaluated by the Tukey test for a confidence interval of 95%. Data were represented as the average ± standard deviation.

3. Results and discussion

3.1. Composition of murta fruit and leaves extracts

The content of some compounds in murta fruit and leaves extracts were quantified by HPLC and the results are shown in the first block of Table 1, and their concentrations were expressed as mg of every compound per gram of fruit or leaves. In agreement with previous works, rutin, quercitrin hydrate and ellagic acid dihydrate were the principal compounds identified and quantified in murta leaves (Avello et al., 2013). Other flavonoids well known by their high antioxidant activity were also identified and quantified, such as isoquercitrin, catechin hydrate, narcissin (isorhamnetin-3-O-

rutinoside) and isorhamnetin-3-O-glucoside, but their concentrations presented low values. Table 1 also includes other chemical compounds identified, and sometimes quantified, by previous studies. It is worthy to mention a recent work done by Peña-Cerda et al., (2017) where 102 different compounds were identified from ethanolic extracts of 10 different genotypes of murta leaves (Peña-Cerda et al., 2017). Table 1 only presents the ten compounds with highest concentration.

Two different extracts of murta leaves were analyzed in order to see the effect of the solubility on the extraction process. Table 1 presents chemical composition of leaves extractions under ethanol 50% (L-E2) and water (L-E3). Although the mixture water-ethanol was able to extract higher total amount of identified components, some compounds were better extracted under water, as it was the case of rutin and quercitrin hydrate. Solubility is strongly affected by the nature of both the solvent and the flavonoid structure (Chebil et al., 2007). The presence of sugar groups is also an important factor. As it was already mentioned in the previous section, non-glycoside flavonols were better extracted with ethanol, as it was the case of catechin hydrate and ellagic acid. On the other hand, concentration of glycoside flavonoids were higher when extraction was carried out using water (shown in Table 1 as E3).

In addition, the presence of these antioxidants was greatly lower in murta fruit extract. Shene et al. studies have also shown the presence of flavonoids were higher in leaves than fruits, and the solution 50% ethanol was the most efficient in extracting polyphenols (Shene et al., 2009). Some studies have shown the presence of other compounds, such as myricetin glucoside and quercetin glucosides, but these compounds were not identified in this work. Shene et. coworkers found the main difference between the polyphenolic profile of fruit and leaves were the presence in the leaves extracts of flavan-3-ols, both in monomeric and in polymeric form (Shene et al., 2009).

As Table 1 shows, the total concentration of identified flavonoids in murta leaves was approximately 3 times higher than the total flavonoid content of murta fruit (2.32 mg antioxidants/ g fruit and 6.81 mg antioxidants/ g leaves). The same tendency presented phenolic content and most antioxidant capacities shown in Figure 1.

3.2. Phenolic content and antioxidant activity results

Fig. 1 presents phenolic content and antioxidant activity results of murta fruit and leaf extracts. Phenolic content values of murta fruit were between 23-34 mg gallic acid/g dry fruit, depending the solvent used. These results were higher than the range of values reported for fruits and other berries, such as plum, blackberries, raspberries and strawberries (Hauser et al., 2014; Rufino et al., 2010; Wang & Lin, 2000). On the other hand, murta leaves extracts presented phenolic values considerably higher than fruit samples (4 times higher approximately) and higher than other leaves (Azlim Almey et al., 2010; Yadav, Khare, & Singhal, 2017). Certainly, all biological properties associated to murta leaves infusions are associated to these high values. It must be taken into account that / Furthermore, it has also been described that flavonoids tend to combine when they are part of a mixture, forming hydrogen bonds that may diminish antioxidant activity or may also compete with each other at scavenging free radicals (Hidalgo, Sánchez-Moreno, & Pascual-Teresa, 2010), Despite this chemical competition, as it was observed in other studies, when extractive solvents were compared, highest values of antioxidant activities were correlated with highest values of phenolic content (Arancibia et al., 2011; Hauser et al., 2014; Peña-Cerda., et al., 2017; Rufino et al., 2010). As Fig. 1A-E show, for both fruit and leaves, hydroalcoholic extracts presented highest values of phenolic content and antioxidant activities due to their chemical composition. Previous works have shown

372 polyphenols in murta fruit and leaves can be divided in two major groups: (i) flavonol
373 glycosides, such as myricetin, kaempferol, and quercetin glucosides (rhamnoside,
374 dirhamnoside, xyloside and glucuronide); and (ii) non-glycoside flavanols, mainly
375 epicatechin. Glycoside flavonoids were better extracted under water, and the compounds
376 with higher hydrophobic character were better extracted with ethanol (Junqueira-
377 Gonçalvez et al., 2015; Rubilar et al., 2006; Shene et al., 2009).

378 Concerning to antioxidant activity values, it is a difficult task to choose the most
379 appropriate method to determine antioxidant capacity. Therefore, it is necessary to
380 perform various methods for a better understanding of antioxidant capacities. FRAP and
381 ABTS methods are generally indicated for hydrophilic compounds. The DPPH method
382 may be employed routinely with aqueous-organic extracts containing hydrophilic and
383 lipophilic compounds. As Fig. 1 shows, results obtained from different methods are not
384 always correlated, mainly when these methods are based on a single mechanism of action,
385 as it was the case of results obtained following ORAC (Fig. 1B) and FRAP (Fig. 1C)
386 assays, which are methods based on proton and electron transference, respectively.
387 Differences in the measurement of antioxidant activity through different assays have
388 already been reported (Dudonne, Vitrac, Coutiere, Woillez, & Merillon, 2009; Zhu, Lian,
389 Guo, Peng, & Zhou, 2011). Interestingly, when results of antioxidants activities measured
390 through FRAP and ORAC assays were compared, fruit extracts presented highest values
391 when activities were measured through FRAP (approximately 70 mg Trolox per gram of
392 fruit), while leaves extracts presented highest antioxidant capacities through ORAC
393 method (approximately 274 mg Trolox per gram of leaves). These results only indicated
394 that compounds of fruit and leaves extracts presented different mechanism of antioxidant
395 action. Chemical compounds from leaves extracts presented preference to transfer proton
396 as antioxidant mechanism. Nevertheless, when total antioxidant activity was measured by

DPPH (Fig. 1E) and TEAC (Fig. 1D) methods, which were able to measure the antioxidant ability by single electron transfer and proton donor mechanisms, both methods presented the same tendency than polyphenolic content results.

3.3. Antimicrobial activity results

3.3.1. MIC and MBC determination results

The evaluation of antibacterial activities of murta fruit and leaf extracts against *E. coli* and *L. monocytogenes* were quantitatively assessed by determining MIC and MBC values. Murta fruit and leaves extracts exhibited all antimicrobial activity against both bacteria. As Table 2 shows, results depended on three factors: i) type of extraction solvent (water and ethanol); ii) type of sample (fruit and leaves); and iii) type of microorganisms (Gram-positive or negative).

Extracts with highest composition of antimicrobial active agents content would present lowest MIC and MBC values. In general, there was a correlation between phenolic content values of different extracts and antimicrobial activities. Hydroalcoholic extracts showed highest antimicrobial activities conducting to the lowest MIC values, excepting the case of the ethanolic murta fruit extract (F-E1´) which presented the highest antibacterial ability against *E. coli.* Fig. 1 already showed ethanolic and hydroethanolic fruit extracts reported similar phenolic content, but probably, some compounds better extracted under ethanol were more effective against Gram-negative. Hauser and coauthors (2014) have studied the antimicrobial activity of murta leaf extracts against *L. innocua,* and the highest antimicrobial activity was also obtained with hydroalcoholic extracts. The count reduction was 3.5 log-cycles for the aqueous and 4.5 log cycles for the 50% ethanolic extract (Hauser et al., 2014).

In addition, as expected from Table 1 data and phenolic content values, antimicrobial capacities of fruit extracts were slightly lower than values obtained with leaf extracts due to the different chemical composition of fruit and leaves. Other studies have also shown that leaves presented higher level of polyphenols, such as flavan-3-ols and flavonol glycosides, and other active agents extracted that could have antimicrobial activity, such as organic acids and small lignin fragments (Shene et al., 2009). Surprisingly, fruit extracts were more effective against Gram negative bacteria, such as *E. coli*, than leaves extracts. This fact was probably due to the presence of some anthocyanins on murta fruit extracts, as shown in other studies (Junqueira et al., 2015). Antibacterial activity of berries and other anthocyanin-containing fruits can be caused by multiple mechanism and synergism between various compounds including anthocyanins, weak organic acids, phenolic acids and their complex chemical mixtures (Cisowska, Wojnicz, & Hendrich, 2011).

As Table 2 shows, and in agreement with previous studies, extracts presented highest antimicrobial activities against *L. monocytogenes*, a Gram-positive bacteria. Gram-negative organism have a major defense system and are less susceptible to the action of many antimicrobial compounds because the presence of an outer membrane surrounding the cell wall that limit the diffusion of hydrophobic substances through its lipopolysaccharide covering (Vaara, 1992). Conversely, Gram-positive bacteria only have a peptidoglycan without external membrane, being the path for the antimicrobial components action is more accessible (Gutierrez, Rodriguez, Barry-Ryan, & Bourke, 2008; Harvey, Champe, & Fisher, 2007). Antimicrobial substances interact with microbial membrane proteins, enzymes and lipids, thereby altering cell permeability and permitting the loss of protons, ions and macromolecules. The mechanism of antibacterial action can be divided in two important aspects: i) inhibition on the synthesis of bacterial

447 nucleic acids; ii) cellular membrane damage causing change on fluidity and exit of
448 intracellular compounds. Other studies have also shown that the presence of bioactive
449 compounds from berries hindered the adherence of bacteria to epithelial cells, which is
450 necessary for colonization, and infection of many pathogens (Gensokowsky et al., 2015).
451 Anthocyanins of cranberry concentrate have also produced a down-regulation of genes
452 encoding outer membrane proteins in *E. coli* O157:H7 (Wu et al., 2013).
453
454 *3.3.2. Cytometry results*
455 Flow cytometry assays were carried out to study the viability of cells exposed to murta
456 extracts. Tests were performed with *Listeria monocytogenes*, since this microorganism
457 showed higher sensitivity to murta extracts (shown in Table 2). SYTO-13 is a
458 fluorochrome able to penetrate all bacterial membranes and bind nucleic acid of the cells.
459 On the other hand, PI penetrates the cell membranes when they are damaged (Rodriguez,
460 Seguer, Rocabayera, & Manresa, 2004). As Fig. 2 shows, the samples were successfully
461 stained. The assay demonstrated that the percentage of damaged cells after treatment with
462 MIC values of hydroalcoholic murta extracts was in accordance with the antimicrobial
463 effect observed in the previous studies.
464 As it can be observed in Fig. 2A, the positive control of *L. monocytogenes* control
465 presented 77.41% of alive cells (stained with SYTO-13) and 21.17% dead bacteria
466 (stained with PI). This value of non viable cells can be explained by the different phases
467 of cellular cycle during the cytometry assay.
468 The damaged of *L. monocytogenes* was evident after treatment with murta extracts.
469 The effect of hydroalcoholic murta fruit extract produced a dead population of 93.21%,
470 as it can be seen in Fig. 2B. After the interaction between the bacteria and the
471 hydroalcoholic murta leaves extract at MIC concentration, the result was found to be

472 97.93% of non-viable cells (Fig. 2C). In this sample, the evaluation of the cytometry assay
473 was very difficult since leaves extract was composed by some components which
474 coloration affected the analysis by the Flow Cytometer. The fact that emission and
475 excitation light of some leaf-extract compounds (probably chlorophylls) were similar to
476 those of the fluorochromes affected the analysis, and the graph obtained was not so clear
477 as with the other samples.
478
479 *3.3.3. Antifungal activity results*
480 Antimicrobial activity of murta extracts against *Penicillium expansum* was studied in
481 liquid medium. Hydroalcoholic extracts of fruit and leaf produced an inhibition growth
482 of (3.79 ± 0.22)% and (4.76 ± 0.30)%, respectively. Although antifungal activity was
483 higher with the leaf extracts, both hydroalcoholic extracts showed low antifungal activity
484 against *Penicillium expansum.* Low antifungal effectiveness of murta fruit and leaf
485 extracts against *Aspergillus niger* and *Penicillium expansum* was already observed by
486 other authors. Climatic conditions during plant cultivation, the different areas of
487 cultivation and the genotype are determining factors in the phenolic profile of murta
488 leaves and fruits. Extreme climatic conditions could increase the phenolic profile of
489 leaves. The influence of all these factors makes it difficult to compare studies between
490 different authors. In addition, it is important to highlight the concentration of these
491 extracts that implied their higher effectiveness against microorganism growth (Alfaro et
492 al., 2013; Ferrer-Gallego, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailon, 2012;
493 Shene et al., 2009).
494
495 *3.4. Anti-tyrosinase activity results*
496

Fig. 3 shows the results of tyrosinase inhibitory activities by the hydroethanolic extracts of murta fruit and leaves, expressed as the concentration necessary to inhibit tyrosinase activity at 50% (CE_{50}). Anti-tyrosinase activities of aqueous extracts were also measured in order to confirm the same tendency observed in other activities. Results confirmed hydroalcoholic extracts of both murta fruit and leaves presented the highest anti-tyrosinase activities, resulting on CE_{50} values approximately 2 to 3 times lower than results obtained from aqueous extracts. It was also clear that murta leaves presented higher tyrosinase inhibitory effect than fruit samples. As it was expected, results followed similar relationship with phenolic content of the extracts. Such as the work done by Sun et al. (2017) in which the anti-tyrosinase activities and concentrations of bound phenolic extracts (BPE) of rape bee pollen exhibited linear correlation. Free phenolic extracts (FPE) of rape bee pollen showed higher activity than BPE. Inhibitory activity against tyrosinase was also observed for some phenolic compounds, such as quercetin, benzoic acid and their derivatives. Therefore, inhibitory tyrosinase activity of FPE and BPE is related to their phenolic profiles. For its part, Kaewnarin et al., (2016) moderate correlation was observed between tyrosinase inhibitory activity of all mushroom species and their phenolic (r = 0.420) and flavonoid (r = 0.557) contents. According to their phenolic profiles, high levels of flavonoid compounds were found, such as catechin, rutin, apigenin and rosmarinic acid. Chang (2009) demonstrated that flavonoid compounds have potential as natural tyrosinase inhibitors. Additionally, rosmarinic acid displayed a strong tyrosinase inhibitory activity on mouse melanoma cell line B16F10 at low concentration (Oliveira, Palu, Weffort-Santos, & Oliveira, 2013). Generally, the number of hydroxyl groups of the phenolic compounds could influence the tyrosinase inhibitory activity that forms a hydrogen bond to the active site of low enzyme leading to the low enzyme activity (Alam, Yoo, & Lee, 2011). Therefore, the presence of compounds with

antioxidant activity may be one of the important mechanisms involved in tyrosinase inhibition (Kaewnarin, Suwannarach, Kumala, & Lumyong, 2016). In addition, several researchers have reported the anti-tyrosinase of various phenolic resources, including soybean, Ornithogalum narbonense, mentha and wild edible mushroom (Fatiha et al., 2015; Kaewnarin et al., 2016; Shukla et al., 2016; Zengin, Uysal, Ceylan, & Aktumsek, 2015).

The variation in activity between fruit and leaves and solvent extraction might be explained based on the chemical composition of extracts and the potential synergistic effect of individual extract components. Tyrosinase inhibitors repress conversion of tyrosinase to DOPA, and subsequently melanin. Thus, tyrosinase inhibitory activity might be dependent on the hydroxyl groups of phenolic compounds, which forms hydrogen bonds with enzyme sites to promote steric hindrance, conformational changes, and suppression of enzymatic activity. Other studies have also shown interesting results of anti-tyrosinase activities of phenolic compounds from natural sources, such as green tea and grape seed (Momtaz et al., 2008; Yamakoshi et al., 2006).

The values obtained for control drugs were CE_{50} (arbutin)> 100 g/L and CE_{50} (Kojic acid)= 35.62 mg/L. As it was already shown in other studies, natural extracts presented pretty low inhibitory activities when compared to kojic acid, but if the comparison is done with arbutin, results were quite interesting, mainly in the case of aqueous leaves extracts (Momtaz et al., 2008).

4. Conclusion

Because evaluation methods of antimicrobial, antioxidant and anti-tyrosinase activities have not been sufficiently standardized yet, making comparisons with other

studies and fruits is difficult. Even though, the data obtained add valuable information to current knowledge on the nutritional properties of murta fruit and leaves, such as an interesting phenolic content, considerable antioxidant and antimicrobial capacities and a promising effect as tyrosinase inhibitor. HPLC confirmed the presence of phenolic acids and flavonoids, and quantification revealed highest concentration in the case of murta leaves extract. Hydroalcoholic extracts of murta leaves were the most promising for the cosmetic industry showing highest anti-tyrosinase activities. Traditional uses of murta fruit and leaves were validated through phenolic content results and antioxidant assays.

New applications of the murta fruit and leaves, naturally occurring or as extract, on functional food, nutraceutical and cosmetic products may be interesting thanks to their antimicrobial, antioxidant and anti-tyrosinase capabilities.

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743

744 **Table 1**

745 Chemical composition of some quantified flavonoids of murta fruit and leaves extracts,
746 expressed as mg of compound per gram of fruit/leaves.

Rt (min)	Compound	F - E2	L- E2	L- E3
9,02	Catechin hydrate	ND	0.57	ND
22,10	Rutin (Quercetin-3-O-rutinoside)	1.30	1.99	2.42
22,35	Isoquercitrin (Quercetin-3-O-glucoside)	0.20	0.63	0.60
23,16	Ellagic acid dihydrate	0.15	2.56	0.80
24,96	Quercitrin hydrate (Quercetin-3-O-rhamnoside)	ND	0.81	1.14
25,37	Narcissin (Isorhamnetin-3-O-rutinoside) + Isorhamnetin-3-O-glucoside *quantified with narcissin as standard	0.67*	0.25*	0.17*
TOTAL COMPOUNDS (mg antioxidants/g sample)		2.32	6.81	5.13

747 Rt: retention time; ND: not detected

748

749 **Table 2**

750 MIC and MBC values expressed in (mg/mL) of murta fruit and leaf extracts.

	Escherichia coli		*Listeria monocytogenes*	
Extract	MIC	MBC	MIC	MBC
F-E1´	1.2	1.7	0.6	0.9
F-E2´	3.8	5	0.2	0.5
F-E3´	8.3	10.0	1.8	2.2
L-E1´	5	6.7	0.2	0.5
L-E2´	4.1	5.5	0.07	0.09
L-E3´	10.3	12.0	0.7	0.9

751

752

753 **Figure captions**

754

755 **Fig. 1**. Phenolic content and antioxidant activity results of murta fruit (F-E1, F-E2, F-E3)
756 and leaf extracts (L-E1, L-E2, L-E3) obtained through different methods: A) Folin
757 Ciocalteu, B) ORAC, C) FRAP, D) TEAC, and E) DPPH assays. Letters a-d indicate
758 significant differences among the extracts of the same method.

759

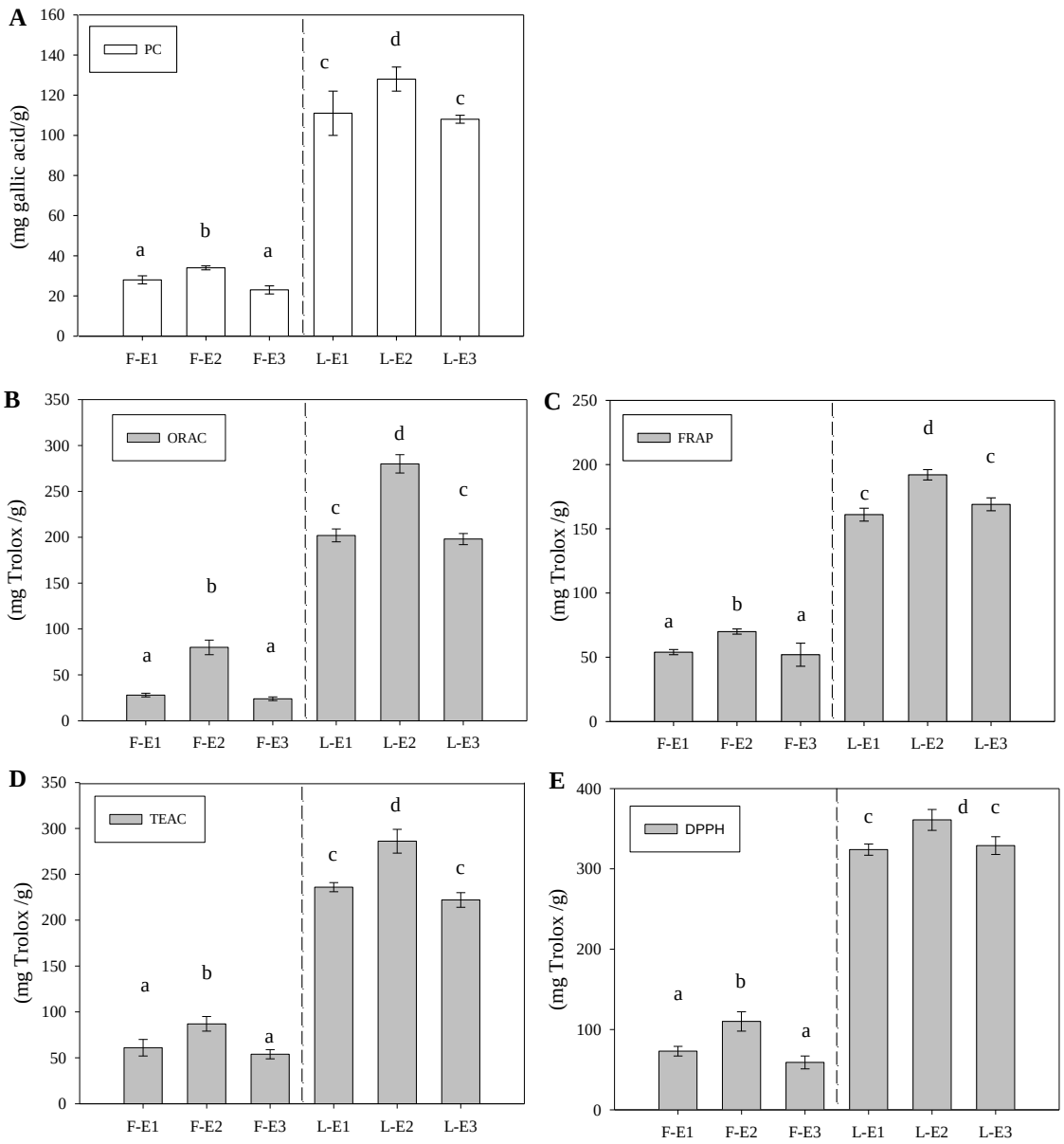
760 **Fig.2.** Dual parameter SYTO-13/PI fluorescence histograms obtained by staining *L.*
761 *monocytogenes*: A) Control samples; B) treated with hydroalcoholic murta fruit extract
762 at MIC; C) treated with hydroalcoholic murta leaf extract at MIC.

763

764 **Fig. 3**. Concentration necessary of hydroalcoholic and aqueous extracts of murta fruit and
765 leaves to inhibit tyrosinase activity at 50%. Letters a-d indicate significant differences
766 among values.

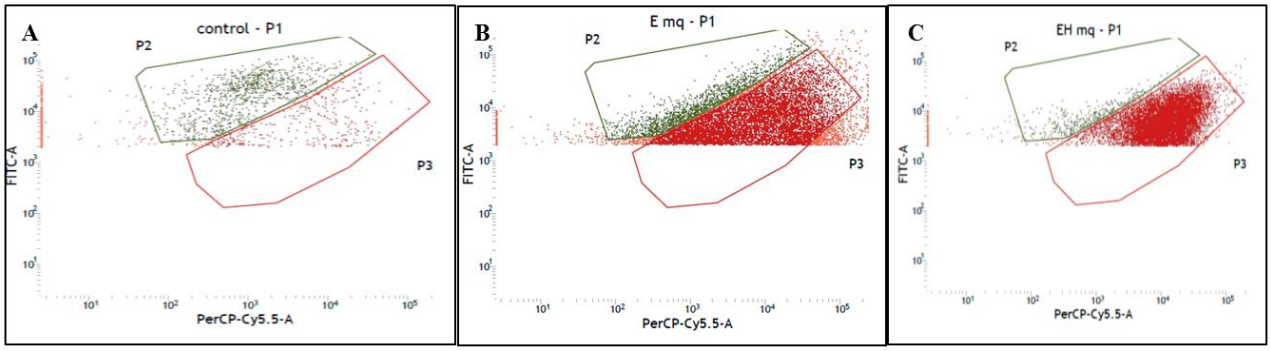
767

768 **Fig. 1**


A
PC
(mg gallic acid/g)
0
20
40
60
80
100
120
140
160
a
b
a
c
d
c
F-E1
F-E2
F-E3
L-E1
L-E2
L-E3
B
ORAC
(mg Trolox /g)
0
50
100
150
200
250
300
350
a
b
a
c
d
c
C
FRAP
(mg Trolox /g)
0
50
100
150
200
250
a
b
a
c
d
c
D
TEAC
(mg Trolox /g)
0
50
100
150
200
250
300
350
a
b
a
c
d
c
E
DPPH
(mg Trolox /g)
0
100
200
300
400
a
b
a
c
d
c

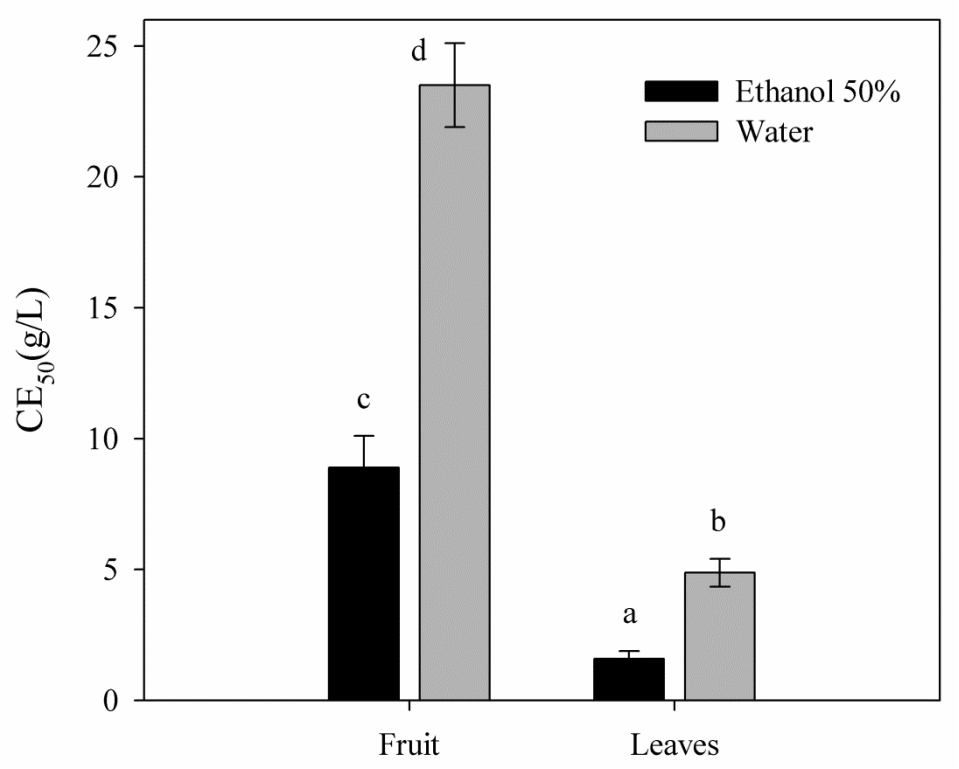
769

770 **Fig.2.**



771

772



773

774 **Fig. 3**.

775

- Buscar referencias de catacterizacion antocianinas murta fruit (Reviewer 1)
- Revisar como se secaron las muestras
- Buscar ref con valores polifenolicos
- Abstract- incluir numerical values

Compounds	Murta fruit			Murta leaves			References
	ethanol 50%	ethanol 85%	water	ethanol	ethanol 50%	water	
Catechin hydrate	ND		2.696		0.57	ND	Jofre et al., 2016; Shene et al., 2012
Rutin (Quercetin-3-O-rutinoside)	1.30	X		X	1.99	2.42	Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017; Shene et al., 2009
Isoquercitrin (Quercetin-3-O-glucoside)	0.20	X		X	0.63	0.60	Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017
Ellagic acid dihydrate	0.15			X	2.56	0.80	Peña-Cerda et al., 2017
Quercitrin hydrate (Quercetin-3-O-rhamnoside)	ND	X			0.81	1.14	Junqueira-Gonçalves et al., 2015
Narcissin (Isorhamnetin-3-O-rutinoside) + Isorhamnetin-3-O-glucoside	0.67				0.25	0.17	
Cafeico ácido-3-glucósido		X		X			Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017
Quercetin		X	0.009	X	X		Jofre et al., 2016; Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017; Shene et al., 2012
Gallic acid		X	0.059	X			Jofre et al., 2016; Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017
Luteolina		X					Junqueira-Gonçalves et al., 2015
Kaempferol		X	0.014				Jofre et al., 2016; Junqueira-Gonçalves et al., 2015
Kaempferol-3-glucósido		X		X			Junqueira-Gonçalves et al., Peña-Cerda et al., 2017; Shene et al., 2012
Luteolina-3-glucósido		X					Junqueira-Gonçalves et al., 2015
p ácido -cumárico		X					Junqueira-Gonçalves et al., 2015
Myricetin		X	0.115	X	X		Jofre et al., 2016; Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017; Shene et al., 2012
Quercetin-3-β-D-glucoside			0.141				Jofre et al., 2016; Junqueira-Gonçalves et al., 2015
Myricetin glucoside	X			X	X		Peña-Cerda et al., 2017; Shene et al., 2012, 2009
Quercetin dirhamnoside	X			X	X		Peña-Cerda et al., 2017; Shene et al., 2009
Quercetin glucoside	X				X		Shene et al., 2012, 2009
Quercetin glucuronide	X			X	X		Peña-Cerda et al., 2017; Shene et al., 2012, 2009
Flavan-3-ol (E)GC					X		Shene et al., 2009
Flavan-3-ol (E)C					X		Shene et al., 2009
Polymeric flavan-3-ol (E)C					X		Shene et al., 2009
Myricetin xyloside				X	X		Peña-Cerda et al., 2017; Shene et al., 2012, 2009
Myricetin rhamnoside				**X**	X		Peña-Cerda et al., 2017; Shene et al., 2009
Quercetin rhamnoside				X	X		Peña-Cerda et al., 2017; Shene et al., 2012
Galloyl-quercetin hexoside				X			Peña-Cerda et al., 2017
Quercetin conjugate					X		Shene et al., 2012
Myricetin conjugate					X		Shene et al., 2012
Myricetin rhamnosyl glucoside				X	X		Peña-Cerda et al., 2017; Shene et al., 2012

Compounds	Murta fruit			Murta leaves			References
	ethanol 50%	ethanol 85%	water	ethanol	ethanol 50%	water	
Catechin hydrate	ND		2.696		0.57	ND	Jofre et al., 2016; Shene et al., 2012
Rutin (Quercetin-3-O-rutinoside)	1.30	X		X	1.99	2.42	Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017; Shene et al., 2009
Isoquercitrin (Quercetin-3-O-glucoside)	0.20	X		X	0.63	0.60	Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017
Ellagic acid dihydrate	0.15			X	2.56	0.80	Peña-Cerda et al., 2017
Quercitrin hydrate (Quercetin-3-O-rhamnoside)	ND	X			0.81	1.14	Junqueira-Gonçalves et al., 2015
Narcissin (Isorhamnetin-3-O-rutinoside) + Isorhamnetin-3-O-glucoside	0.67				0.25	0.17	
Cafeico ácido-3-glucósido		X		X			Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017
Quercetin		X	0.009	X	X		Jofre et al., 2016; Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017; Shene et al., 2012
Gallic acid		X	0.059	X			Jofre et al., 2016; Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017
Luteolina		X					Junqueira-Gonçalves et al., 2015
Kaempferol		X	0.014				Jofre et al., 2016; Junqueira-Gonçalves et al., 2015
Kaempferol-3-glucósido		X		X			Junqueira-Gonçalves et al., Peña-Cerda et al., 2017; Shene et al., 2012
Luteolina-3-glucósido		X					Junqueira-Gonçalves et al., 2015
p ácido -cumárico		X					Junqueira-Gonçalves et al., 2015
Myricetin		X	0.115	X	X		Jofre et al., 2016; Junqueira-Gonçalves et al., 2015; Peña-Cerda et al., 2017; Shene et al., 2012
Quercetin-3-β-D-glucoside			0.141				Jofre et al., 2016; Junqueira-Gonçalves et al., 2015
Myricetin glucoside	X			X	X		Peña-Cerda et al., 2017; Shene et al., 2012, 2009
Quercetin dirhamnoside	X			X	X		Peña-Cerda et al., 2017; Shene et al., 2009
Quercetin glucoside	X				X		Shene et al., 2012, 2009
Quercetin glucuronide	X			X	X		Peña-Cerda et al., 2017; Shene et al., 2012, 2009
Flavan-3-ol (E)GC					X		Shene et al., 2009
Flavan-3-ol (E)C					X		Shene et al., 2009
Polymeric flavan-3-ol (E)C					X		Shene et al., 2009
Myricetin xyloside				X	X		Peña-Cerda et al., 2017; Shene et al., 2012, 2009
Myricetin rhamnoside				**X**	X		Peña-Cerda et al., 2017; Shene et al., 2009
Quercetin rhamnoside				X	X		Peña-Cerda et al., 2017; Shene et al., 2012
Galloyl-quercetin hexoside				X			Peña-Cerda et al., 2017
Quercetin conjugate					X		Shene et al., 2012
Myricetin conjugate					X		Shene et al., 2012
Myricetin rhamnosyl glucoside				X	X		Peña-Cerda et al., 2017; Shene et al., 2012

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Compounds	Murta fruit			Murta leaves			References
	ethanol 50%	ethanol 85%	water	ethanol	ethanol 50%	water	
Catechin hydrate	ND		2.696		0.57	ND	a, e
Rutin (Quercetin-3-O-rutinoside)	1.30	X		X	1.99	2.42	b, c, d
Isoquercitrin (Quercetin-3-O-glucoside)	0.20	X		X	0.63	0.60	b, c
Ellagic acid dihydrate	0.15			X	2.56	0.80	c
Quercitrin hydrate (Quercetin-3-O-rhamnoside)	ND	X			0.81	1.14	b
Narcissin (Isorhamnetin-3-O-rutinoside) + Isorhamnetin-3-O-glucoside	0.67				0.25	0.17	
Cafeico ácido-3-glucósido		X		X			b,c
Quercetin		X	0.009	X	X		a, b, c, e
Gallic acid		X	0.059	X			a, b, c, e
Luteolina		X					b
Kaempferol		X	0.014				a,b
Kaempferol-3-glucósido		X		X			b, c, e
Luteolina-3-glucósido		X					b
p ácido -cumárico		X					b
Myricetin		X	0.115	X	X		a, b, c, e
Quercetin-3-β-D-glucoside			0.141				a, b, c, e
Myricetin glucoside	X			X	X		c, d, e
Quercetin dirhamnoside	X			X	X		c, d, e
Quercetin glucoside	X				X		c, d
Quercetin glucuronide	X			X	X		c, d, e
Flavan-3-ol (E)GC					X		d
Flavan-3-ol (E)C					X		d
Polymeric flavan-3-ol (E)C					X		d
Myricetin xyloside				X	X		c, d, e
Myricetin rhamnoside				X	X		c, d
Quercetin rhamnoside				X	X		c, e
Galloyl-quercetin hexoside				X			c
Quercetin conjugate					X		e
Myricetin conjugate					X		e
Myricetin rhamnosyl glucoside				X	X		c, e

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