EFFECT OF THE BENCH SCALE EXTRACTION CONDITIONS ON *Pinus radiata* BARK EXTRACT YIELD, ANTIOXIDANT PROPERTIES AND COMPOSITION

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ABSTRACT

Many pine bark extracts have antioxidant properties. This work examined bench scale extraction on the yield, the composition and antioxidant properties of *Pinus radiata*. Acetone solvent (98.7% w/w at 120 °C) yielded maximum phenols concentration, tannins concentration, anti-radical activity (DPPH) and reducing activity (FRAP), although its extraction yield was the lowest. When the organic solvent in the mixture decreased, the chelating activity increased (ICA) and antiradical and reducing activities decreased. The extract largest yield was obtained with water at 120ºC, but the antioxidant properties were relatively poor. Taxifolin (dihydroquercetin) and catechin were the main extract components detected by RP-HPLC. The best extraction conditions for high extractive yield and antioxidant properties was achieved with ethanol/water at 120 °C.

Keywords: Antioxidant, bark, bench scale, extraction conditions.

INTRODUCTION

Lipid oxidation in food can be prevented with the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and ter-butyl hydroquinone (TBHQ), but the potential carcinogenic properties of these antioxidants limits their use in some countries (Botterweck et al. 2000, Rupasinghe *et al.* 2010). Thus, natural antioxidants to food lipids are increasingly studied (Kähkönen *et al.* 1999, Fukumoto and Mazza 2000, Rahim *et al.* 2008).

The forest and paper industry generates large amounts of organic wastes of different compositions (Jerez *et al.* 2007a), where pine bark generated in the production process of wood pulp for paper usually is combusted or wasted although several extractable compounds could be used as high value products. Multiple authors have reported the antioxidant bioactivity of pine bark extract (Jerez *et al.* 2006, Ku *et al.* 2007, Cortés *et al.* 2010, Dudonné *et al.* 2009). In comparison with other pine species, *Pinus radiata* bark has shown the highest values for polyphenol content and antiradical activity (Jerez *et al.* 2007a, Ku *et al.* 2007).

Many studies of natural antioxidant extraction from pine (Jerez *et al.* 2007a, Jerez *et al.* 2006, Ku *et al.* 2007, Jerez *et al.* 2007b), grape (Kennedy and Jones 2001, Kennedy and Taylor 2003), apple (Rupasinghe *et al.* 2010) and plants (Heimler *et al.* 2007, Romani *et al.* 2006) have been performed, although most have been conducted on a laboratory scale. Indeed, Jerez *et al.* (2006, 2007b), Ku *et al.* (2007) and Diouf *et al.* (2006) used 0.16 L reactors, 0.25 L extractors and 22 cm^3 cells, respectively;

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meanwhile other authors (Kennedy and Jones 2001, Kennnedy and Taylor 2003, Rupasinghe *et al.* 2010, Heimler *et al.* 2007, Romani *et al.* 2006) used scales ranging from 30 to 1000 mL for the extraction of various plants.

Usually, it is difficult to obtain a satisfactory correlation between the extraction variables at laboratory and pilot scales; and larger extraction trials are recommended (Treybal 2001). Therefore, in the present study, pine bark extraction was performed using a 4 L extractor (bench scale) and a more realistic approach to obtain the process parameters to scale up to pilot and industrial scale extraction processes.

The objective of this work was to study bench scale extraction with different solvents and temperatures, and measure yields, composition and antioxidant properties. For all cases, anti-radical, reducing and chelating activities of the extracts were measured; phenols, tannins extracts, average molecular weight and phloroglucinolysis yields were determined; and the extract components were identified through comparison with standards.

MATERIALS AND METHODS

Chemicals and reagents

Standards of arabinose, ascorbic acid, (+)-catechin hydrate, galactose, glucose, mannose, taxifolin and xylose were used in the HPLC analysis were purchased to Sigma-Aldrich (St. Louis, MO, USA); as well, butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrous chloride, ferrozine, Folin-Ciocalteau reagent, gallic acid, iron III chloride, linoleic acid, phloroglucinol and 2,4,5-tri(2-pyridyl)-s-triazine solution (TPTZ). Acetone, acetic acid, ethanol, hydrochloric acid and methanol were analysis grade; sulfuric acid and acetonitrile were HPLC grade and they were purchased to Merck (Darmstadt, Germany); as well as, sodium acetate, ferrous sulfate and sodium phosphate. Pygnogenol® (50 mg) was purchased to Prater Laboratory (Concepción, Chile). Also, ammonium sulphate (Sharlau, Barcelona, Spain), ammonium thiocyanate (Equilab, Santiago, Chile), methyl cellulose (BD Falcon, Franklin Lake, NJ, USA) and sodium carbonate (Arquimed, Santiago, Chile), were used in this study.

Preparation and extraction

P. radiata bark was obtained from the San Luis Farm, Yungay, Chile (May, 2010). Pine bark was ground with a mill (Condux, Hamilton, Canada) and classified with a sieve to a size 20 ± 5 mm diameter (Williams 16-31, Concepción, Chile), dried to room temperature until obtaining equilibrium humidity (~12%), and then maintained at 23 ± 1 °C in a room. Extraction was carried out in a 4 L reactor (P.Faudler, Schwetzingen, Germany) using 2 L of solvent and 100 g of pine bark (dry weight). After reaching operating temperature (40 °C, 120 °C or 160 °C), the time was initiated for 2 h. Using a filter of 150 μm, the wet bark was separated from the solution. The solution was then evaporated using a rotary evaporator (Ika Werke, Staufen, Switzerland) at 40 °C, to eliminate the solvent used in the extraction. The aqueous extract was filtered using a Whatman paper filter No.1 (11 mm) to separate the water-soluble polyphenol fraction from the non-soluble particulate material at room temperature. Finally, the water-soluble polyphenol portion was freeze-dried (Labconco, Kansas City, MO, USA) at -50 °C and 0.05 mbar, and kept in amber glass containers, sealed at -20 °C.

Extraction conditions

A preliminary assessment of the effect of the temperature on the extraction yield and extract properties was studied. Some extractions were performed at higher temperatures (120 °C and 160 °C) than the boiling temperature of the solvent and so had pressures above atmospheric.

After that, the effect of the organic solvent concentration on the extraction yield and extract properties was studied. Table 1 shows the extraction conditions to determine the solvent's influence on the pine bark extract yield at two temperatures (T_1=40 ºC and T_2=120 ºC).

Table 1: Batch extraction conditions to determine the solvent and temperature influence on the pine bark extract yield. Solvent/bark ratio: 20 (mL/g, w/w).
Extraction time: 2 hours.

Solvent/water	% w/w
Water	
Ethanol/Water	25.0 ± 0.2
Ethanol/Water	50.0 ± 0.2
Ethanol/Water	75.0 ± 0.2
Ethanol/Water	98.7 ± 0.2
Acetone/Water	25.0 ± 0.2
Acetone/Water	50.0 ± 0.2
Acetone/Water	75.0 ± 0.2
Acetone/Water	98.7 ± 0.2

Extraction yield
The results are expressed as % grams of extract per gram of dry bark.

Extract's moisture content and solubility
To determine moisture, 50 g of extract were dried at 102 °C, according to Belscak-Cvitanovic *et al.* (2010) For solubility analysis, the extract was dissolved in 10 mL of distilled water at 30 °C, stirring occasionally during 30 min; the liquid was centrifuged (Heraeus Biofuge Primo, Frankfurt, Germany) at 5348 g for 10 min, the supernatant was poured, and the solution was left at 105 °C until constant weight.

Total phenol content on the extracts
The Folin-Ciocalteau method was performed as reported by Singleton and Rossi (1965) with modifications by Jerez *et al.* (2006). A pine extract sample of 0.5 mL (0.1 g/L) was mixed with 2.5 mL of Folin-Ciocalteau reagent (2 N) diluted at 10% (v/v) and 2.0 mL of 75 g/L of sodium carbonate. The solution was incubated for 15 min at 45°C and its absorbance was measured in a glass cuvette (path length 10 mm) at 765 nm with a spectrophotometer (Shanghai Techcomp Instrument Ltd., Shanghai, China). A blank solution was prepared following the previous methodology, but replacing the extract with 2.5 mL of Milli-Q™ water. A calibration curve was performed using 0-60 mg/L of gallic acid aqueous solution as standard. Total phenol concentration of the extracts was expressed as the equivalent of mg of gallic acid/g of dry extract.

Pine bark extracts tannin's concentration
A methyl cellulose precipitation assay developed by Sarneckis *et al.* (2006) for grape extract and adapted by Maldonado (2008) for pine bark extract was used. Firstly, 0.5 mL of an aqueous solution of extract (1.0 mg/mL) was mixed with 1.5 mL of a 0.04% (w/w) aqueous solution of methyl cellulose (0.5 mL, 1 g/L). Subsequently, 1.5 mL of a saturated solution of ammonium sulphate (55 g/mL), and 1.5 mL of Milli-Q™ water were added and the sample was kept for 15 min at room temperature. The mixture

was centrifuged at 2218 g for 5 min and the absorbance of the supernatant was measured at 280 nm. The blank solution followed the same methodology, but replacing the methyl cellulose solution with 1.5 mL of Milli-Q™ water. Results were quantified by a calibration curve of (+)-catechin and expressed as equivalents of milligrams of (+)-catechin per gram of extract.

DPPH assay

The antiradical activity of the extracts was expressed as the percentage of antiradical activity (%AA), measured by the sequestration of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Ku and Mun (2008) and by Yassa *et al.* (2008). For the sample, 1 mL methanolic extract solution (12 mg/L) was mixed with 2 mL of methanolic solution of DPPH (0.1 mmol/L). For the blank, 1 mL of methanol was mixed with 2 mL of 0.1 mmol/L methanolic solution of DPPH, whereas for the control, 1 mL of methanolic extract solution (12 mg/L) was mixed with 2 mL of methanol. After 30 min in the dark, the absorbance was measured at 518 nm with the spectrophotometer. The antiradical activity was determined as $\%AA=(1-((Abs_m - Abs_c)/Abs_b))*100$, where %AA stands for antiradical activity shown by the sample, while Abs_m, Abs_c and Abs_b correspond to sample, control and blank absorbances, respectively.

Ferric reducing/ antioxidant power (FRAP) assay

The extract's reducing activity was measured using the FRAP assay as described by Tian *et al.* (2009). The solution, 2,4,5-tri(2-pyridyl)-s-triazine (TPTZ) of 10 mmol/L, was prepared in 40 mmol/L of hydrochloric acid. The FRAP solution was prepared mixing 10 volumes of sodium acetate buffer (pH 3.6, 0.3 mol/L), 1 volume of TPTZ solution and 1 volume of iron(III) chloride 20 mmol/L solution. Analyses were performed mixing 3.6 mL of FRAP solution preheated at 37 °C, 360 µL of Milli-Q™ water and 120 µL of 100 mg/L methanolic solution of bark extract. Then, the mixture was incubated at 37 °C for 30 min in a temperature-controlled bath (Heidolph Instruments, Schwabach, Germany). The auto-zero of the spectrophotometer was made with acetate buffer; sample absorbance was measured at a wavelength of 595 nm. A reagent blank was prepared, using the same procedure as for the samples, but replacing the extract with 120 µL of methanol. Results were expressed in units of equivalent g Fe^{+2}/g extract by means of a calibration curve prepared with a methanolic solution of ferrous sulfate with a concentration range of 0.2 to 2.5 mmol/L.

Iron chelating activity (ICA) assay

The chelating activity of obtained polyphenolic extracts was determined by ICA method (iron (II) chelating activity assay) according to Maqsood and Benjakul (2010). Firstly, 4.4 mL of extract methanolic solution (2 g/L), 100 µL of $FeCl_2$ (1 mmol/L) and 200 µL of ferrozine (5 mmol/L) in Milli-Q™ water were mixed. Samples were incubated for 20 min at room temperature and subsequently centrifuged (2218 g x 5 min). A reagent blank was made following the same procedure as for the samples, using 4.4 mL of distilled water instead of the extract solution. Furthermore, a blank sample was prepared following the previously described procedure, replacing the $FeCl_2$ solution with distilled water. Sample absorbance was determined at a wavelength of 562 nm for all samples.

Lipid peroxidation and ferric thiocyanate method (FTC)

Lipid peroxidation and peroxide production's inhibition by the extracts was measured with the FTC method (Ku and Mun 2008, Larrauri *et al.* 1996). The pine extracts (0.8 g/L) were diluted with absolute ethanol. Linoleic acid (65%) was diluted in ethanol up to 2.5% w/w. The extract solution (7 mL) was mixed with 14 mL of sodium phosphate (0.3 mol/L, pH 7.0), 7 mL of deionized water and 7 mL of 2.5% linoleic acid. Control solution was prepared by mixing 14 mL of sodium phosphate, 7 mL of deionized water, 7 mL of 2.5% linoleic acid and 7 mL of absolute ethanol. The lipid peroxidation was placed in a tube with a screw cap, and placed in an oven at 40°C in the dark. Then, 100 µL of the lipid/sample solution was mixed with 75% ethanol (9.7 mL) and 30% ammonium thiocyanate (50 µL). Precisely 3 min after addition of 0.1 ml of 0.02 mol/L ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance was measured against a reagent blank at 500 nm, each 24 h, until the control reached maximum. Results were expressed as % inhibition of the primary peroxidation (%IPP).

Sugar analysis by HPLC

The sugar content of the extract produced with acetone 75% (w/w) at 40°C and at 160°C was measured by injection of the extracts using a HPLC, following the procedure by Mendonça *et al.* (2008). A sample (300 mg) was weighed in a test tube and 3 mL of 72% (w/w) sulfuric acid was added. The hydrolysis was carried out in a water bath at 30 °C for 1 h with stirring every 10 min. Later, the acid was diluted to 3% (w/w) with 79 mL of distilled water and the mixture was transferred to a 250 mL Erlenmeyer flask and autoclaved for 1 h at 121 °C. The residual material was cooled and filtered through a number 3 sintered-glass filter. Solids were dried to constant weight at 105 °C and determined as insoluble lignin. Soluble lignin was determined by measuring the absorbance of the solution at 205 nm. The concentration of monomeric sugars in the soluble fraction was determined by HPLC with refractive index detector (Merck Hitachi, Tokyo, Japan) and a column HPX87H (Biorad, Hercules, CA, USA) at 45 °C, eluted at 0.6 mL/min with 5 mM sulfuric acid and using a refractive index detector. The results were expressed as g of glucose/L, g of xylose+mannose+galactose/L and g of arabinose/L.

Proanthocyanidins analysis by HPLC

Proanthocyanidins of extracts were depolymerized by acid hydrolysis in presence of phloroglucinol (Kennedy and Taylor 2003, Cortés *et al.* 2010). RP-HPLC (Younglin, Shanghai, China) equipped with a RP-18E column, 3.0 x 150 μm, 5 μm (Merck Chromolith, Darmstadt, Germany) and a UV-VIS detector (Younglin, Shanghai, China) were used for gradient liquid chromatography. The extract was mixed in an ultrasound bath with 10 mg/L of a solution, containing 0.1 mol/L hydrochloric acid in methanol, 50 g/L phloroglucinol and 10 g/L ascorbic acid. Then, the mixture was filtered through 0.45 μm (Membrane Solutions, Lane Plano, TX, USA), and 20 μL were injected into RP-HPLC. The method used a binary gradient with A: water containing 1% (v/v) aqueous acetic acid and B: acetonitrile containing 1% (v/v) acetic acid. Eluting peaks were monitored at 280 nm, and the elution conditions were as follows: column temperature 30 °C; 3.0 mL/min; 3% B for 4 min, a linear gradient from 3% to 18% B in 10 min, and 80% B for 2 min. The parameters were calculated using the 100 mg/L standards of (+)-catechin and taxifolin. The average molecular weight (aMW) and the phloroglucinolysis yield (%PY) were calculated (Kennedy and Jones 2001).

Statistical analysis

All the extract's chemical antioxidant analysis and the HPLC characterization data were the results of the means from three independent replicates ($n = 3$). All data are reported as mean value ± SD. The calculation of mean values and standard deviation was performed by Microsoft Excel® 2003.

RESULTS AND DISCUSSION

Preliminary assessment of the temperature effect on the extraction yield

Highest yields were achieved with water, reaching 11.99% at 120 °C ($p<0.05$). The extracts obtained at 160 °C were dense, viscous, caramelized and presented a non-homogeneous composition, which would cause pipe plugging at commercial plant scale. At 160 °C, eventually polysaccharide hydrolysis and subsequent dehydration to furans occurred but this was not measured.

The sugar content of two extracts obtained with an acetone/water mixture at 40 °C and at 160 °C, respectively, were analyzed and the results are presented in Table 2. The extract obtained at 160 °C had more sugar concentrations than obtained at 40 °C, explaining its larger density and viscosity. Therefore, and due to this extract's physical characteristics, the maximum operation temperature was limited to 120°C.

Table 2. Sugar analysis of pine bark extracts obtained with an acetone/water mixture as solvent.

Extraction temperature (°C)	Composition of extract	Concentration (g/L)
40	Glucose	0.710 ± 0.007
	Xylose+Mannose+Galactose	0.095 ± 0.001
	Arabinose	0.009 ± 0.001
160	Glucose	0.550 ± 0.004
	Xylose+mannose+galactose	1.400 ± 0.009
	Arabinose	1.500 ± 0.013

Extraction conditions: acetone/water 7/3 (v/v), solvent/ bark ratio= 20 mL/g, extraction period = 2h.

Extraction Yield

Figure 1 shows the bark's extraction yields for the different organic solvent at 40 and 120ºC. No caramelization was observed in these products. The yields at 40 °C were smaller than one half of the yields at 120 °C. The largest yields were obtained using water, which shows that polar solvents produce the highest extraction. To choose the best extract, however, information related to their physicochemical and antioxidant properties is needed.
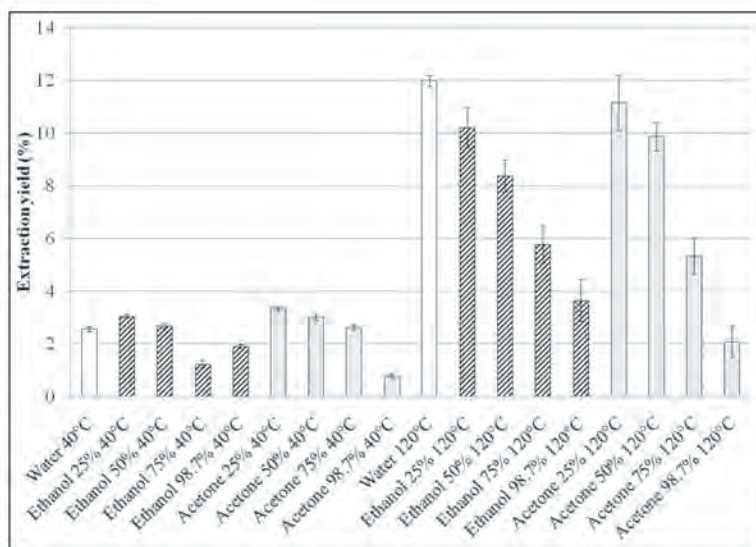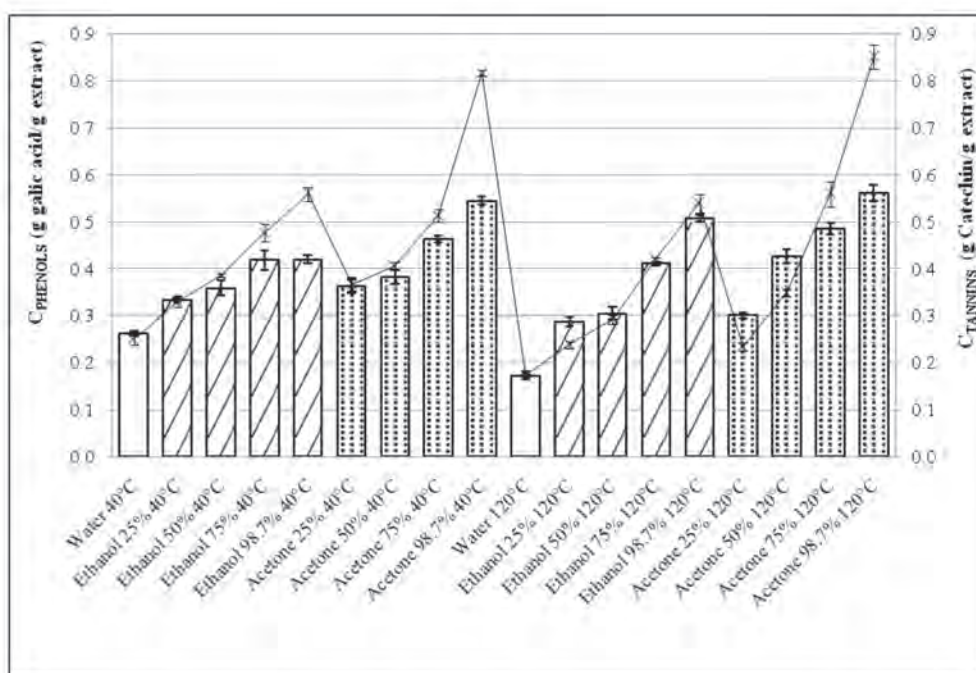

Figure 1. Effect of temperature and organic solvent concentration (Acetone: ⋮⋮ Ethanol: ◿ and Water:☐) on the pine bark extraction yield. Extraction period= 2 h; solvent/ bark ratio (v/w) =20 mL/g.

Extract's moisture content and solubility

The moisture and solubility of the extracts were determined to evaluate the influence of the extraction solvent. The extract obtained with water had the highest moisture content (12.1%), probably due to absorption of water molecules from the environment because of its higher hidrophilicity. In contrast, the six extracts obtained with the ethanol/water (25, 50 and 75% w/w) and acetone/water (25, 50 and 75% w/w) mixtures did not differ significantly, showing average moistures of 7.6±1.2%. Extract solubility did not show significant differences between the extracts with a mean value of 17.5±0.2 g/L.

Total phenols and tannins concentration

Increased temperature causes a decrease in total phenols and tannin concentrations (Figure 2) except for the extractions with high organic solvent content (75% and 98.7%), i.e., it was possible that extract denaturation occurred at 120 °C. The extraction at 120 °C with water had a $C_{PHENOLS}$: 0.172 g of gallic acid/g extract; this concentration was smallest than all other extracts. The highest total phenols concentration was obtained with acetone 98.7% at 40 ºC and 120 °C. The same behavior was observed for the tannins concentration. Pinelo *et al.* (2005), also obtained a larger total phenols concentration when ethanol instead of water was used as solvent.

Figure 2. Effect of temperature and organic solvent concentration on the total phenol content (Acetone: ⁞⁞ Ethanol: ⧄ and Water:☐) and tannin's concentration (✳) of pine bark extracts.

Antioxidant properties

It is difficult to evaluate the antioxidant activity of a compound based on a single method due to the complexity of mechanisms involved in oxidation reactions (Viuda-Martos *et al.* 2010). Therefore, in this work, many methods such as those that measure anti-radical activity (DPPH), reducing (FRAP) and iron chelating (ICA) activities, and peroxide production's inhibition in linoleic acid (FTC) were evaluated to characterize pine extract antioxidant capacity.

Anti-radical, reducing and iron chelating activities

Many researchers report anti-radical activity for pine extracts, although reducing and chelating activity is scarcely reported. It is important to measure the reducing activity or ferric reducing/antioxidant power (FRAP) because phenols antioxidant power are considered to be related to their redox properties, which act as reducing agents (Kähkönen 1999). Also, the ability to form molecular complexes with heavy metals is very important for fat oxidation since these are capable of catalyzing the oxidation process (Lantto *et al.* 2009). Thus, in order to determine antioxidant properties, antiradical, reducing and chelating activities all should be determined.

Figure 3 shows the antiradical, reducing and chelating activities of the 18 studied extracts. The anti-radical activity of the extracts increased when the organic solvent percentage was raised, reaching the highest value of 41 %AA with acetone (98.7% at 40°C and 120°C). Ku *et al.* (2007) reported values of 92.5% of anti-radical activity, working with *P. radiata* extracts (25 μg/mL), equivalent to 44.4% of AA in the extract concentrations used in our work. For *P. radiata* bark extracts (0.15 g/L) produced with acetone, Maldonado (2008) reported 86 %AA at 35 °C, equivalent to 6.88% of antiradical activity using the extract concentration reported in our work. The extracts presented here are similar to those of Ku *et al.* (2007), who also found good antiradical activity.
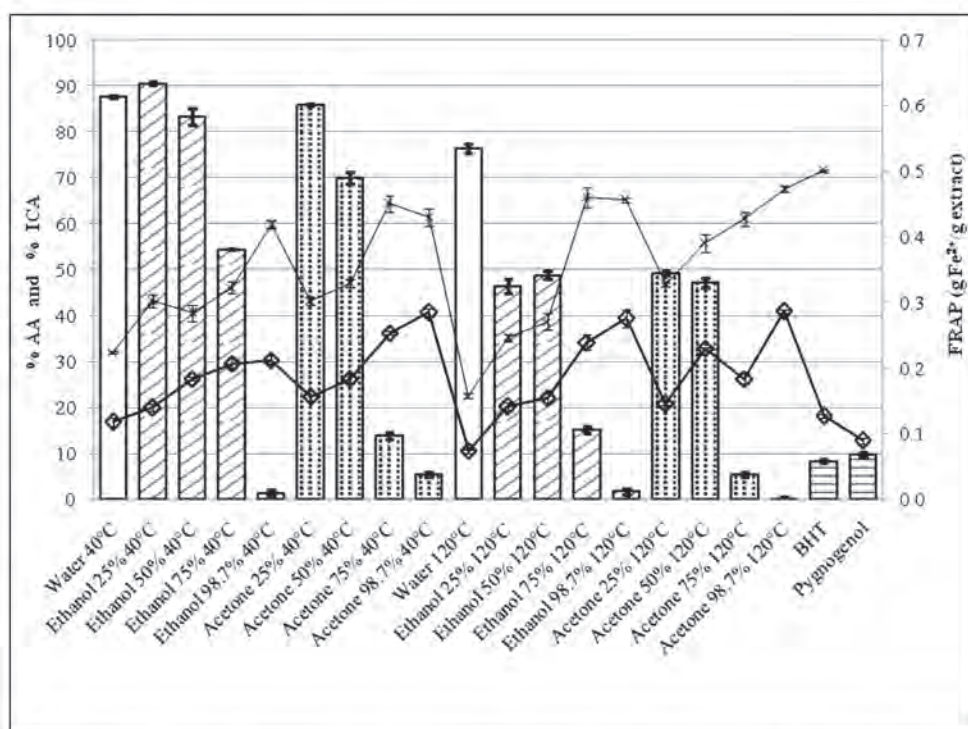
Figure 3. Effect of temperature and organic solvent concentration on the iron chelating activity % ICA (Acetone: ⁝⁝, Ethanol: ▨, Water:☐, and commercial antioxidants ≡), antiradical activity %AA (◇) and ferric reducing/antioxidant power or FRAP (✳) of pine bark extracts.
Extraction period: 2 h; solvent/ bark ratio (v/w) = 20 mL/g.

A tendency similar to the antiradical activity was observed for the experimental reducing activity data (FRAP), where the maximum value was 0.47 g Fe^{2+}/g extract for the extracts obtained with 98.7% of acetone at 120 °C. The behavior of both antiradical and reducing activities reveals that the compounds responsible for the free radical scavenging and reduction are more easily extracted with solvents less polar than the water. Several extracts decreased their antiradical and reducing activity when the extraction temperature was raised to 120 °C. Comparable results were reported using Galla chinensis extracts (Tian *et al.* 2009).

Similar to the anti-radical and reducing activity, ICA values decreased when the extraction temperature increased; nevertheless, the effect of the type of organic solvent and the mass proportions on ICA were different. Indeed, ICA decreased when the proportion of organic solvent increased, showing no chelating activity for the extract obtained with 98.7% acetone at 120 °C. This phenomenon has been observed when non-polar solvents are used to extract pine bark. Indeed, the effect of these compounds on metals is inhibited due to the activity of the metoxi groups without affecting their antiradical and reducing activity (Maqsood and Benjakul 2010 and Jerez *et al.* 2009). Jerez *et al.* (2009) have reported the presence of ferulic acid rich in metoxi groups when *P. radiata* bark was extracted with 99.5% ethanol at 37.5 ℃. This result could explain the low chelating activity and high antiradical/reducing activities when high organic solvents are used.

Low ICA values for the extracts obtained with a high ratio of organic solvent were similar to the ICA values for the commercial antioxidants: BHT and Pygnogenol. Both commercial antioxidants showed ICA values equal or lower than 10%. The extracts obtained with a solvent concentration < 75% showed higher values of %AA, FRAP and ICA than the commercial antioxidants.

Measurement of the inhibition of peroxide production in linoleic acid

For the extracts with the best extraction yield and anti-radical, reducing and chelating activities, the inhibition of primary lipid peroxidation in linoleic acid (IPP) was measured. The best performance was for BHT (IPP of 97.42%), followed by extract obtained with water (IPP of 92.6%). One cause for this behavior could be the water affinity of extracts in the solution of linoleic acid/ water solution leading to a larger dissolution of the extracts, and therefore a better action and results. However, the extracts obtained with organic solvents also showed high IPP values; for example, extracted obtained with 50 and 75% of ethanol registered IPP of 87.9 and 81.7% respectively.

Relation between extract's properties

The anti-radical and reducing activities of the extracts presented a direct relationship ($R^2= 0.829$). Similar behavior was reported (Jerez *et al.* 2007a) for plants and pine extracts ($R^2= 0.822$). Thus, when an extract shows high antiradical activity, it also exhibits high reducing activity. Also a linear relationship between the experimental values of DPPH and total phenols ($R^2= 0.884$) was found. Thus, phenols have influence of on the antiradical activity of the extracts and on the possible antioxidant activity since a larger number of phenolic groups implies a larger group of hydroxyl groups, which are potentially capable of trapping free radicals. In pine bark extracts, these groups are mainly provided by proanthocyanidins. The relationship between anti-radical activity and tannins concentration was also direct ($R^2= 0.726$). Thus, it is believe that not only tannins but a combination of many types of phenols are responsible for antiradical activity.

Extraction yield-antioxidant activity optimization

When the organic solvent to pine bark ratio (v/w) was increased, extracts total phenols and tannins concentration increased. Also, anti-radical and reducing activity behavior was similar, while chelating activity and lipid oxidation decreased. When the temperature was raised from 40 °C to 120 °C, there was also a decrease in the measured extract properties and at the same time an increase in the extraction yields; although, the increase in the extraction yield was larger than the decrease of the former properties. Since both, the extract antioxidant properties and the extraction yield have to be considered when choosing the most attractive extract, the results were expressed as a product of both properties (Table 3).

Table 3 shows that the most attractive extractions were done with water at 120 °C; 25% and 50%, ethanol at 120 °C, and 25%, 50% and 75% acetone at 120 °C. The main advantages of the extract obtained with water at 120 °C are high yield, chelating activity and inhibition of primary lipid oxidation; nevertheless, this extract had the lowest antiradical and reducing activity, phenols and tannins concentration, i.e., their bioactive properties are low when compared to other extracts (Ku *et al.* 2008, Pinelo *et al.* 2005, Lantto *et al.* 2009).

When acetone was the extraction solvent, yields were similar to those obtained with ethanol but the extracts showed higher values of anti-radical and reducing activity, phenols and tannins concentration. Thus, these could be the best extracts, although acetone handling is considered riskier than ethanol usage (according to National Institute for Occupational Safety and Health and the American Conference of Governmental Industrial Hygienists). In addition, if extracts are to be used in food, ethanol is more recommended as extracting agent (Shi *et al.* 2005). In conclusion, the best extracts for flexible use and their properties are those obtained with 25% and 50% ethanol.

This study shows that the use of different extraction process conditions generates extracts with diverse chemical and physical characteristics. Wu *et al.* (2011), have determined that the chelating activity is an important characteristics of anticancer agents, and therefore those extracts with high ICA and low values of %AA and FRAP are potential anticancer agents. On the other hand, those extracts obtained with a high organic proportion (high %AA and FRAP, and low values of ICA) can be used as antioxidant agents in food and paint industries.

Proanthocyanidins analysis by HPLC

The 18 extracts were characterized using RP-HPLC after catalysis acid with phlorglucinol. Two adducts, epicatechin-(4β-2)-phoroglucinol (EC-P) and catechin-(4α-2)-phoroglucinol (C-P), and two monomers, (+)-catechin (C), taxifolin (T), were detected and quantified. Retention times were in accordance with results previously published (Cortés *et al.* 2010).

Figure 4 shows retention times of 10 g/L extract obtained at 40 °C with 75% acetone. The 18 extracts had the same compounds. After component identification, the phloroglucinolysis yield (%PY) and the average molecular weight (aMW) were determined. Figure 5 shows that %PY increases with the increase in organic solvent proportion. For both temperatures, acetone as solvent produced larger yields than ethanol or water. The increase in temperature was detrimental to the performance, which agrees with the antioxidant characterization of the extracts (Figure 3) and their phenol and tannins concentration.

The extraction temperature was the parameter that most influenced the aMW: when it was raised from 40 to 120 °C, the aMW decreased 44%. The aMW ranged between 552 and 1251 g/mol, similar to those reported for pine bark extract using GPC (Weber *et al.* 2007).

Taxifolin identification led to the discovery that the phenolic compounds had the highest concentration, followed by (+)-catechin. This identification was not confirmed in previous reports (Cortés *et al.* 2010).
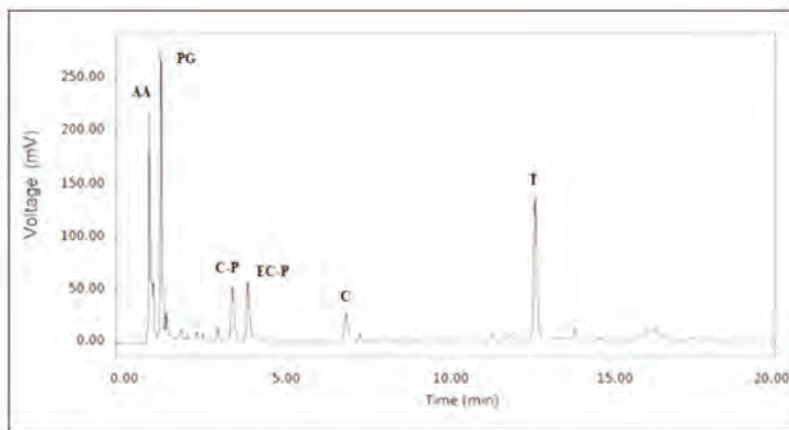
Figure 4. Chromatogram from batch extract obtained with 75% (w/w) acetone at 40°C, solvent/bark ratio= 20 mL/g; extraction period of 2 h. AA: Ascorbic acid; PG: Phloroglucinol; C-P: Catechin-Phloroglucinol adduct; EC-P:Epicatechin-Phloroglucinol adduct; C:(+)-Catechin and T: Taxifolin.
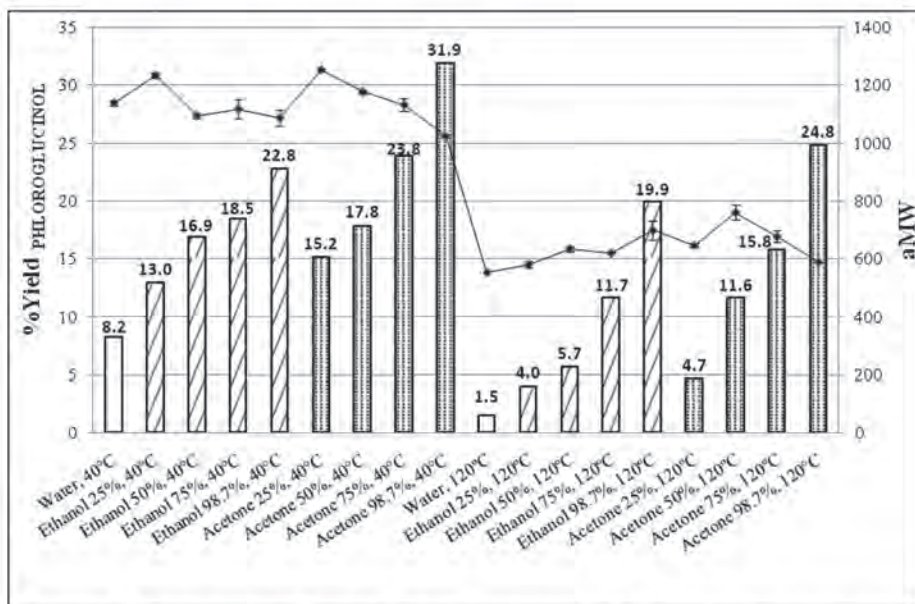
Figure 5. Temperature and organic solvent concentration effect on phloroglucinolysis Yield %PY (Acetone: ⁝⁝ Ethanol: ⁒ and Water:☐) and average molecular weight aMW (◆) content in extracts from pine bark.
Extraction period= 2 h; solvent/bark ratio (v/w): 20 mL/g.

CONCLUSIONS

Phenols bench extraction from pine bark was performed with different process conditions and the extracts were characterized in terms of an extraction optimization. At extraction temperature of 160 °C, caramelization and non-homogeneous physical characteristics were observed, making scaling up at 160 °C infeasible. When the temperature decreased from 120 to 40 °C or when the organic solvent ratio was increased, extraction yields decreased and larger phenols and tannins concentration and bioactivities were observed. The anti-radical activity of the extracts increased when the organic solvent percentage was raised. The behavior of both antiradical and reducing activities reveals that the compounds responsible for the free radical scavenging and reduction are more easily extracted with solvents less polar than the water. The best extraction conditions for high extractive yield and antioxidant properties was achieved with ethanol/water at 120 °C. The chromatographic information indicated that the extract's main components were catechin and taxifolin.

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