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COMPARISON OF PHENOLIC EXTRACTS OBTAINED OF PINUS RADIATA BARK FROM PULP AND PAPER INDUSTRY AND SAWMILL INDUSTRY

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ABSTRACT

Pinus radiata barks obtained from tress of different ages, as subproduct of pulp and paper (trees less than 10 years) and sawmill (trees between 20 to 25 years) industries, were used to produce extracts containing phenolic compounds. A factorial design 2^3 was used to evaluate the influence of the variables temperature (25 – 35 ºC), solvent type (acetone - ethanol) and extraction time (1-12 h). The extracts were compared in their extraction yield (%), total phenols (by FolinCiocalteau), and radical scavenging activity (by DPPH). The extract obtained from old trunks presented a higher extraction yield than from young trees. The highest yield value was 2.56%, which was obtained using acetone as solvent for 12 h and 35°C. The highest concentration of phenol (5.84±0.18 g CE g extract^{-1}), and scavenging activity (IP=86.1±4.4%) were also obtained for this type of extract. The extraction duration was the variable that most influenced the parameters studied. The bark's radical scavenging power was greater than BHT (40%) and slightly lower than ascorbic acid (92%), common commercial antioxidants.

Keywords: Antioxidants; bark; *Pinus radiata*; tannins.

INTRODUCTION

The forest industry is an important productive sector based on renewable natural resources. As a result, considerable amounts of products are discarded each year, mainly from sawmill and cellulose industries as processing byproducts that include sawdust, wood chips and barks (Egaña 1999). Statistics revealed that the total amount of wood residues produced during 2000-2001, from the main exported countries, exceeded 6 million of cubic meters (EFI 2003). Although, the statistics do not clearly indicate their final destinations, most of the byproducts were used for particle boards or energy purposes (Hillring 2006). These recycled products have low economic value, even when a number of bioactive compounds have been identified in bark (Balaban and Ucar 2001, Nunes *et al.* 1999). Therefore, there exists a great potential for the forest industry to convert and utilize more of these byproducts, transforming them into high value products.

In Chile, 67.8% of wood production is from *Pinus radiata*; of this percentage, 32% correspond to pulp and paper production, 40% to sawmill wood and the rest to others uses. The length, diameter and age of the trees used for cellulose and sawmill wood production are different (Maldonado 2008), and consequently the properties and characteristics of their bark should differ.

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Koreans and Spanish researchers have reported the extraction of phenols from *Pinus radiata* bark and their potential use in the food industries for to their antioxidant properties (Jerez *et al.* 2006; Jerez *et al.* 2007a; Jerez *et al.* 2007b; Raghavendra *et al.* 2007). Also, antioxidant effects have been described for extracts from *Pinus maritime* bark (Pycnogenol®) (Packer *et al.* 1999) and from *Pinus pinaster* (Jerez *et al.* 2007a) under diverse extraction conditions.

Barks extract is a natural resource that could replace synthetic antioxidants as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT), which are suspected of being responsible for liver damage and carcinogenesis in laboratory animals (Nishioka *et al.* 2007). Recently, evidence has demonstrate that common pine (*Pinus sylvetris* L.) bark extracts (Sokol-Letowska *et al.* 2007) and mangrove *Rhizophoraapiculata* bark extracts (Rahim *et al.* 2008) had an efficacy similar or superior to BHT, BHA and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid (Trolox) at equivalent conditions.

The extraction protocols for solvent extraction should be an effective technique to obtain the highest yield extraction and adequate bioactives properties of the extract. These parameters are highly dependent on the extraction conditions, as evidenced for the different values obtained for extraction yield and radical scavenging activity from pine barks (Jerez *et al.* 2006; Palma *et al.* 2003; Raghavendra *et al.* 2007). Therefore, comparative studies to select the most appropriate extraction technique are necessary for each substrate. In a previous work, we produced pine bark extracts with one type of solvent, in a fixed time and temperature and they were chemically characterized, being the major constituent proanthocyanidins (Cortes *et al.* 2010). In this new investigation is seeking to evaluate the influence of some process variables on the extraction; mainly, in the yield extraction and the phenolic content of the extracts. In the future, others variables and technologies of extraction could be studied and the best results will be chemically fully characterized.

The goal of this research was to compare the yield of the extraction in percent, phenolic concentration, and radical scavenging activity of *Pinus radiata* barks extracts obtained from young trees and old trunks processing by Chilean forestry industries. A 2^3 factorial design was performed for each substrate in order to determine the influence of the solvent, extraction time, and temperature on the phenolic extracts features. The bark radical scavenging capacity was also compared with typical commercial antioxidants (BHT and ascorbic acid).

MATERIALS AND METHODS

Bark Samples

Two bark types were used: one from the cellulose industry (young bark) and the other from the sawmill industry (old bark). The young bark was obtained from Arauco Cellulose plant, Arauco, VIII Region, Chile; the age of trees was less than 10 years. The old bark was obtained from Laurel Sawmill, Santa Juana, VIII Region, Chile; the age of trees was between 20 to 25 years. Both samples were dried at room temperature, grounded with a knife mill and sieved to select the particles between 1 and 2 mm. The bark's initial humidity was constant during the experiments (14 ± 1 %) and it was determined by using the weight of bark before and after oven dried at 105°C for 2 h. The bark density for both samples was also determined using a pycnometer.

Experimental Design

Two full factorial 2^3 experimental designs (Box 2000) were developed to evaluate the effect of the temperature (T), extraction duration/time (t) and solvent type (S) in the extracts from barks of young and old trees. Temperature values were 25°C and 35ºC, extraction duration time 1h and 12 h, and the solvents were acetone and ethanol from Merck (Darmstadt, Germany). Statgraphics

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Plus for Windows 4.0 (Herndon, VA) was used for statistical analyses of experimental design. The variables were codified in the way that their value ranged between +1 and -1. Acetone and the highest values of the variables (35ºC and 12 h) were codified as +1; ethanol and the lowest values of the variables (25ºC and 1 h) were codified as -1 for the computational analysis with Statgraphics A central point in triplicate was codified as 0 and represents a central point of variables. The liquid-solid ratio was maintained in1:5 (w/v).

The data were adjusted to a response surface R

$$R = a_o + a_1 t + a_2 T + a_3 S + a_{12} tT + a_{13} tS + a_{23} TS \qquad (1)$$

where a_o is the value of the objective function in the central point conditions, a_1, a_2, a_3 represent the principal effects associated with each variable and the others represent the crossed effects among variables.

Bark extraction

The phenolic extraction from bark was performed in 500-mL capped flasks, where 50 g of ground pine bark (wet base) were solvent extracted at the temperature and for the time specified in the experimental design, in an orbital shaker G24 New Brunswick Scientific Co. Inc. (NJ, USA) with temperature control and at constant stirring rate (200 rpm). Solids were separated by filtration, the solvent evaporated under reduced pressure and temperature (T<40ºC), and the crude extracts reconstituted in 50 mL of bi-distillated water. The lipophilic compounds of the crude extract were removed with a triple hexane wash (1:1 %w/w). The aqueous extracts were freeze-dried and kept at 4ºC for further analysis. The extraction yield (%) was determined as the amount of extract recovered in mass compared with the initial amount of bark in dry base.

Total phenols

Total phenols were determined using the Folin-Ciocalteu method (Singleton and Rossi 1965). 2.5 mL of tenfold diluted Folin– Ciocalteu reagent (Sigma, St Louis,MO, USA), 2 mL of 7.5% sodium carbonate(Sigma, St Louis, MO, USA), and 0.5 mL of phenolic extract (0.15 g/L) were mixed well. After heating the sample for 15 min to 45 ºC, the absorbance was measured at 765 nm (Shimadzu UV-1203, Japan). A mixture of water and reagents was used as blank. A calibration curve was built using (+)-catechin as standard to quantifying of catechin equivalents (mg CE/L) and normalized by the amount of extract obtained in each experimental point to mg CE/g extract.

Radical scavenging activity

To determine the radical scavenging ability of the extracts, a 2,2-Diphenyil-picrylhydrazyl (DPPH) (Sigma, St Louis, MO, USA) radical assay was performed (Brandwilliams *et al.* 1995; Jerez *et al.* 2006).A volume of 980 µL of6.1 10^{-5} M. $DPPH^{\cdot}$ in methanol solution was used. There action was started by adding 20 µL of methanolic crude extract (0.15 g/L). The bleaching of $DPPH^{\cdot}$ was followed at 515 nm until that absorbance remained unchanged (15 min) in the dark and at room temperature. The radical scavenging activity was expressed as the inhibition percentage (IP) of the $DPPH^{\cdot}$ radical and was calculated as:

$$IP(\%) = \left(1 - \frac{\text{absorbance}_{\,t=\text{equilibrium}}}{\text{absorbance}_{\,t=0}}\right) \cdot 100 \qquad (2)$$

The performance of phenolic extract was also compared with commercial antioxidants, butylated hydroxytoluene (BHT) and acid ascorbic, under the same concentration (0.15 g/L).

Statistical analysis

The samples were analyzed in triplicate and the data presented as mean ± standard using the Statgraphics Plus for Windows 4.0 (Herndon, VA).

RESULTS AND DISCUSSION

In Chile, tree diameter and length determine whether it will be used in the sawmill industry or in pulp and paper industry. Trees with a minimum width of 18 cm and a length of 3.2 m with ages from 20 to 25 years are used in sawmills to produce wood, while trees with a diameter minimum of 8 cm and 2.44 m in length (produced during the trimming, thinning and pruning stages) older than five years are used to produce cellulose (CMPC 2008). These differences in raw material suggest variations in the bark's characteristics and composition in physical and chemical terms, as will be established in the next paragraphs.

The physical characteristics of bark obtained from pulp and paper industry and bark from sawmill industry were different. The bark from pulp and paper industry was thinner and ductile with a maximum thickness of 1 cm, while the bark from sawmill industry was thicker and rigid with a maximum thickness of 3 cm. These differences were also represented in their densities. After grinding, young bark density was 580 kg/m^3 and it was lower than old bark density, close to 890 kg/m^3.

Yield extraction of bark from young and old trees

From the data set of experimental design, the amount of extract obtained in relation to the amount of bark processed, for both barks (dry base) is presented table 1 and table 2, respectively. The old bark presented a higher yield (10% in average) than young bark yield. The analysis of each set of experiments shows that the yield was always higher for 12 hours of extraction, with an average difference of 15% with respect to 1 h. The highest yield of 2.56% was obtained for old bark using acetone as a solvent during 12 h extraction at 35ºC.

Table 1. Levels of total phenols, tannins and anti-radical activity for bark extracts from old trees based in a design factorial.

Experiment	Temperature (°C)	Solvent type	Time (h)	Yield (%)	Total phenols (g CE*·g extract^{-1})	Inhibition Percentage (%)
1	25	Acetone	12	2.49	5.34 ± 0.01	82.9 ± 5.8
2	35	Acetone	12	2.56	5.44 ± 0.04	79.1 ± 4.7
3	25	Ethanol	12	2.46	4.90 ± 0.13	73.7 ± 4.7
4	35	Ethanol	12	2.43	5.46 ± 0.08	80.4 ± 4.3
5	25	Acetone	1	2.11	5.67 ± 0.03	83.4 ± 3.8
6	35	Acetone	1	2.01	5.84 ± 0.18	86.1 ± 4.4
7	25	Ethanol	1	2.10	5.63 ± 0.04	83.4 ± 1.4
8	35	Ethanol	1	2.21	5.44 ± 0.16	83.2 ± 1.3
9	27.5	Acetone	5.5	2.23	5.68	83.2
10	27.5	Acetone	5.5	2.37	5.70	82.7
11	27.5	Acetone	5.5	2.28	5.67	80.5

*CE: Catechin equivalent

The maximum yield obtained in this study was low and similar to other reported for *Pinus radiata* bark (2.0 %); although, with different operating conditions (extraction time 24 h and, solvent Met OH/H_2O (7:3 % v/v)) (Diouf *et al.* 2006). Using more aggressive conditions (NaOH/H_2O 1%, 100ºC), Ku and Mun (2007) increased ten times the yield. This difference could be explained by a probable higher lignin extraction generated by the NaOH addition and higher temperature. Also, by means of successive extractions with hexane, ethyl alcohol and hot water yielded the highest total extractives for pine 43.8% (Hafizoglu *et al.* 2002). The differences with these authors are that in this work, the experiments were designed to be performed at a reduced temperature, with a solvent that can be easily recovered and that will preserve the extract's characteristics.

Table 2. Levels of total phenols, tannins and anti-radical activity for bark extracts from young trees based in a design factorial.

Experiment	Temperature (°C)	Solvent type	Time (h)	Yield (%)	Total phenols (g CE*·g extract^{-1})	Inhibition Percentage (%)
1	25	Acetone	12	2.12	5.49 ± 0.01	79.4 ± 3.5
2	35	Acetone	12	2.13	5.23 ± 0.01	79.9 ± 4.6
3	25	Ethanol	12	2.12	4.93 ± 0.14	77.7 ± 3.6
4	35	Ethanol	12	2.16	5.08 ± 0.13	78.5 ± 3.0
5	25	Acetone	1	1.91	5.20 ± 0.03	84.2 ± 3.0
6	25	Acetone	1	1.92	5.57 ± 0.02	85.9 ± 4.0
7	25	Ethanol	1	1.94	5.25 ± 0.04	79.0 ± 3.5
8	35	Ethanol	1	2.08	5.27 ± 0.22	81.0 ± 4.2
9	27.5	Acetone	5.5	2.18	5.15	81.4
10	27.5	Acetone	5.5	2.11	5.33	81.0
11	27.5	Acetone	5.5	2.08	5.49	82.5

*CE: Catechin equivalent

Characterization of the extracts

The major constituents in pine bark extracts from *Pinus pinaster* and *Pinus massoniana* (Weber *et al.* 2007) and *Pinus radiata* (Cortes *et al.* 2010) are proanthocyanidins. However, given the complexity of their analysis, the composition and properties of the extracts were analyzed according to the total phenol, and radical scavenging activity as indicators of the effect of change the extraction variables.

The characteristics for both barks are presented also in table 1 and table 2, respectively. A lower amount of total phenols and radical scavenging activity were observed in young bark than in old bark. These differences could be associated to a higher accumulation of extractives in aged trees. Changes in the chemical composition of the tree as consequence of the ageing had also been reported in others species such as maritime pine (Esteves *et al.* 2005), *Eucalyptus globulus* (Miranda and Pereira 2002) and larch (Gierlinger and Wimmer 2004).

The highest values of total phenols (5.84±0.18 g CE/g extract), and radical scavenging capacity (86.1±4.4%) were obtained for the operation condition of 1 h, acetone, 35ºC. Considering the differences in the extraction method, quantification methodology and data reports, it is difficult to compare our results with values obtained for *Pinus radiata* bark extracts in other studies. Still, total phenols content was found to be higher than the values obtained for other *Pinus radiata* analyzed with the same analytical method (Diouf *et al.* 2006, Raghavendra *et al.* 2007).

Factorial Analysis

The factorial analysis for the bark extracts from old trees showed that the yield extraction is favored when the variables temperature, contact time and solvent are in their low level (Table 3). The concentration of phenols showed the same behavior, i.e. acetone as solvent, 25ºC of temperature and a contact time of 1 h instead of 12 h, would increase the phenol concentration. The inhibition percentage of the radicals by the extract, was not favored by prolong time of extraction in the interval of variation assayed. The analysis of bark extracts from young trees showed that the temperature was the most influencing variable for the yield and the inhibition percentage. An increase of phenols concentration was favored by the use of acetone as solvent. The models adjusted for the prediction of phenol composition of bark extracts from old trees presented a better adjusted (given by the correlation coefficient, r^2) than the models for bark extracts from young trees.

Table 3. Models obtained from factorial design analysis for the barks analyzed

Variable	Old bark model	Young bark model
Yield	Y=3.52-0.14S-0.15t-0.13S·t (r^2=0.837;F=2.27)	Y=2.06-0.85t (r^2=0.807;F=2.62)
Total phenol	TP=5.52-0.136S-0.148t-0.128S·t (r^2=0.834;F=2.23)	TP=5.27-0.12S (r^2=0.612;F=1.44)

Although, precautions were taken to avoid air or light oxidation, the phenols and tannin concentration seems to decline in a longer extraction time. A decrease in the amount phenols in others substrates, as in wine, also have been reported for a longer extraction period (Waterhouse 2002).

Several solvent systems have been used to extract antioxidants from bark. The solvent systems included hot water extraction (Raghavendra *et al.* 2007, Packer *et al.* 1999), alkaline extractions water/NaOH (2%) (Fradinho *et al.* 2002), ethanol (Jerez *et al.* 2006), acetone/water (70:30 %v/v) (Rahim *et al.* 2008), ethanol/toluene (68:32 %v/v) (Lomax *et al.* 1994), among others. The results of antioxidant activity alsohave been diverse: from 36.4 % at 2 µg/ml until 98 % at 100 µg/ml depending on the extract type and grade of purification (Raghavendra *et al.* 2007). Thus, a general criterion for establishing the best solvent to obtain a high antioxidant capacity is complex. Zhao and Hall (2008) assayed different solvent combinations to extract from raisins, finding that the antioxidant capacity was stronger for 80% - 100% acetone; although, the extraction yield was lower than for other solvent systems (Zhao and Hall 2008). Also, lower molecular weight was present in the ethanol fraction from bearberry leaves, while higher molecular weight was present in the acetone fraction, which had more antioxidant than the ethanol fraction (Pegg *et al.* 2003). In our experience, a higher inhibition percentage of $DPP^{\cdot}$ radical was obtained using acetone as extraction solvent in comparison with ethanol.

Relationship among the phenol concentration and radical scavenging activity

In old bark extract, a direct relationship between total phenols determined by the Folin-Ciocalteu assay and the radical scavenging activity was founded (r^2 =0.820). Ku *et al.* (2007) in hot water bark extracts obtained from different Pines did not find a relationship between total phenols and anti-radical activity; we observed the same tendency in young barks extracts (r^2= 0.202).

The main difference of our study with others studies reported in the literature is that the solvents used (100% acetone and ethanol) have different extraction capacity from that of hot water on the extract's composition: less impurities as sugars are expected and this difference can positively influence the Folin-Ciocalteu assay (Raghavendra *et al.* 2007). Also, the removal of waxes and lipids (with a triple hexane wash) helped mainly to the oxidation of phenols for the DPPH and Folin-Cicalteu assays.

The radical scavenging activity of the extract was compared with commercial antioxidants, BHT and ascorbic acid. Bark extract performed twice as well in comparison with BHT, a chemical antioxidant used in the food industry (86%vs.40%, respectively) and similar to the ascorbic acid (86% vs 92%), a recognized commercial antioxidant. Considering that the BHT and the ascorbic acid presented a purity superior to 99%, and the phenolic extracts were not purified, this performance is an advantage from a process point of view.

Nevertheless, the yields obtained in this work are too low to be considered in an industrial process. In the future, it will be necessary to increase the extraction yield, using other extraction technologies or process conditions, while trying to preserve the high anti-radical activity found in the phenol extract of *Pinus radiata*. These results together with future results can be used to assess their potential use of the bark phenolic extracts in industrial applications.

CONCLUSIONS

This work shows that the tree age influences the physical and chemical characteristics of bark extracts. Young bark collected as sub-products of pulp and paper industry had lower extraction yields, phenol and radical scavenging activity than old bark collected as sub-products of sawmill industry. The factorial analysis shows that the extraction time is the significant variable and a reduced time will help to avoid the phenols oxidation. The acetone extraction increases the anti-radical activity than ethanol extraction. A direct relationship between phenols and the radical scavenging activity was observed. The radical scavenging activity of the extracts produced in this study had similar or better values than typical commercial antioxidants. The bark extracts from old trees have a higher potential to be a natural antioxidant than bark from young trees.

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