Tannins extraction from *Pinus pinaster* and *Acacia dealbata* bark with applications in the industry

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**ABSTRACT**

The use of wood waste is considered a valuable resource and a market opportunity for new products that society and industry can employ. In this context, it seems important to consider the use of the bark of two species that are found in large quantities within the extensive forest area of Galicia (north-western Spain). These species are widely used in industrial processes in which a large amount of waste is generated, mainly bark. In this work, we extracted the tannins from the bark of *Pinus pinaster* and *Acacia dealbata*, and we studied the yields obtained according to three variables: the extraction method applied, soxhlet and in natural conditions with temperature and agitation; the particle size of the samples; and the two solvents used, ethanol and distilled water. The chemical properties of the extracts were also analysed. The yields obtained ranged from 3.6 to 25.8 wt% for *P. pinaster*, and from 5.2 to 29.5 wt% for *A. dealbata*. Phenol and anthocyanin content ranged from 217.8 to 873.2 mg/g extract for *P. pinaster*, and from 77.3 to 820 mg/g extract for *A. dealbata*. Our results demonstrate that the differences in the extracts content depending on the method and particle size show different features of the antioxidant capacity.

1. Introduction

In recent years, the numerous and different ways of taking advantage and using forest biomass resources has attracted the interest of industrial, energy and environmental sectors (Balboa Murias, 2005). Industry and small-scale business use and process raw materials in order to obtain final products with greater added value (Hetemäki and Hurmekoski, 2016; Sathre and Gustavsson, 2009). This includes paper, furniture and lumber (Hyttinen et al., 2002). Through these processes waste is generated, in many cases as a result of the misuse of the raw material (Santos Ucha, 2013). According to Kosseva (2009), millions of tons of organic forest waste are generated every year (Kosseva, 2009), and most of this waste is mainly discarded (Varelas and Langton, 2017). However, wood waste can be considered as a valuable resource (Lykidis and Grigorou, 2008). To satisfy the increase of energy dependence and reduced demand of fossil fuels, several studies have shown how biomass waste can be used as feedstock for combustion (Lu et al., 2017; Rodriguez et al., 2020), as potential source of food for insects (Varelas and Langton, 2017), and to extract and use tannins to produce leather (Pizzi, 2019).

Wood waste refers to products such as bark, woodchips and sawdust (Burton et al., 2003). These by-products can be classified as first or second transformation waste (M’hamedi et al., 2017), and they all have different value depending on their quality and form (Hassan et al., 2019; Mantau, 2015). The first transformation generates waste composed of bark, branches, stubs, leaves and needles and generally, the treatment received by this residual biomass is controlled burning up, in-situ clearing, and as a biomass source for biofuels (Velázquez-Martí, 2006). The waste generated in the industry of sawing, boards, pulp, and second transformation are generally of high quality, due to their densities and low humidity (Elorza et al., 2004).

Tree barks such as those from pines and acacias are an important source of tannins (Filgueira et al., 2017; Kemppainen et al., 2014; Ogawa and Yazaki, 2018). Tannins are compounds found in most vascular plants and produced in most parts of the plant (Pizzi, 2019).

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although some authors have showed that greater presence of tannins is located in the bark than in other part of the trees such as the branches (Barberis et al., 2012; Brunet et al., 2003). Scientific research has shown the importance of this family of compounds, but due to the great diversity of origins and subfamilies that it comprises, it has always been difficult to find the appropriate definition of the term (Santos Ucha, 2013). Currently, the definition that has shown the greatest acceptance by the scientific community is Bate-Smith and Swain’s definition (Bate-Smith and Swain, 1962). Tannins have a phenolic structure and can be divided into condensed and hydrolysable tannins (Shirmohammadi et al., 2018). Commercial hydrolysable tannins are limited due to their higher price (Shirmohammadi et al., 2018). However, condensed tannins, which can be found in species such as pine and acacias, represent 90 % of the world production of tannins (Pizzù, 2003).

Tannins have many applications in different sectors: treating emergent contaminants such as those produced by a Fenton process, as a conditioner in sludge dewatering (Ge et al., 2019), removal and recovering of polluting ions such as copper and cadmium from alkaline aqeous solutions (Sun et al., 2019), removal of antibiotic resistant-bacteria from urban wastewater (Grehs et al., 2019), removal of microalgae from eutrophic reservoirs (Álvarez et al., 2021), development of adhesives for plywood (Matsumae et al., 2019), and bone and skeletal reconstructions (Abdalla et al., 2020). Due to their antioxidant and antimicrobial characteristics, tannins are also considered, by the pharmaceutical industry, as an alternative therapy for inflammatory associative diseases (Abu Zarin et al., 2016). However, the insufficient knowledge regardless the best approach to extract tannins from different species is one of the main issues of using these compounds (Sousa et al., 2019). This lack of knowledge is mainly due to their high variability in their structure and chemical composition (Sousa et al., 2019).

At present, the most common commercial tannins comes from the bark mimosa (Acacia mearnsii or mollissima) and pine trees (Pinus radiata), the wood of quebracho (Schinopsis balansae and Schinopsis lorentzii) and the shoots and leaves of Gambier shrubs (Uncaria gambir) (Khusdami et al., 2019; Wu et al., 2020). The amount of tannins obtained depends on their botanical origin, the extraction process (atomization, lyophilization, evaporation) the solvent used (ethanol, water) and the extraction time (Álvarez, 2007). There are other pines (Pinus sp.), that are also important in the production of tannins (Peña Rodríguez, 2014). The 69.4 % of Galician land (north-western Spain) constitutes forest area, making it the most densely wooded region in Spain (Marey Pérez et al., 2006). This region has a primarily Atlantic biome, characterized by large artificial forests (Seijo, 2005), containing predominaently fast growing species such as Pinus pinaster, Eucalyptus globulus and Quercus robur (Balboa Murias, 2005). By 1987, 500.000 ha of new forests had been planted, 15 % of all Spain reforestation (Seijo, 2005). On the other hand, since the beginning of the 20th century, the forest industry in Galicia has undergone a constant and continuous growing, becoming one of the main industries in the country (Rico Boquete, 2014). Nearly half of the wood production of Spain is carried out in Galicia, 47 % in total (Chas et al., 2002; Picos, 2018). This accounts for 1.9 % of the round-wood production in the European Union (Picos, 2018). The forest industry in Galicia had a turnover of 2200 million euros in 2017 and provided employment to 20,320 people (Picos, 2018; Vecino et al., 2018).

In this paper, two extraction methods were applied to obtain the tannins presented in the barks of pine (P. pinaster) and mimosa (A. dealbata), in a box with a soxhlet and extraction with heat and agitation. The extraction yields were obtained and the chemical properties of the extracts were analysed. Due to the large amount of waste generated in Galician pine forests when this species is cut down for timber use, it would be interesting to analyse the feasibility of reducing the waste, aiming towards a circular economy. In addition, Acacia dealbata, a native to Australia, is one of the most abundant invaders in north-west Spain, especially in conservation areas (Lorenzo et al., 2010). Vazquez de la Cueva (2014), studied the expansion of A. dealbata in some areas of Galicia as a consequence of forest fires (Vázquez de la Cueva, 2014), and he observed that in some areas up to 62.4 of the surface area was covered by A. dealbata in pure or mixed stands. In other areas, up to 33.8 % of the surface area was composed exclusively by A. dealbata. This species has been officially classified as invasive by the Government of Spain (BOE, 2013). In Galicia, following regulations of the European Parliament (Regulation, 2014), a plan for exotic invasive species was put in place (). This plan establishes measures for the prevention and eradication of A. dealbata. Therefore, what it is proposed here, would be the next step to these measures, the exploitation of the resources from an environmental point of view.

2. Material and methods

2.1. Selection of material

In order to carry out this research, bark samples were collected from selected A. dealbata and P. pinaster trunks. The bark samples were chosen in the field from homogenous masses, within the same area to avoid different characteristics in soil and climate conditions. The feet selected were all between 10-15 years old, and with the better growth and quality. The collection height was 1.5 m from the tree stump.

2.2. Preparation of samples

The samples were initially weighted and then were introduced into the P-SELECTA 2002961, a drying oven with circulation by reinforced air, at a constant temperature of 100 ± 5 °C. They were weighed daily at the same time of the day to observe the weight variation from one day to the next. This was done over ten days, when weight stability was achieved. After drying the samples, they were crushed in a knife mill grinder and then sieved using three sieves with different apertures: 0.25 mm, 0.5 mm and 1 mm.

2.3. Tannin extraction

The extraction of tannins was performed using two different methods: extraction with a soxhlet, and extraction through heat and agitation. The variables taken into account for this work were the species used (A. dealbata, P. pinaster), the type of solvent (distilled water, ethanol-water 80/20), the method of extraction (soxhlet, magnetic stirring with heat) and the size (<0.5 mm, 0.5–1 mm and >1 mm) of the solid particles. All extractions for each variable were made by triplicate.

2.3.1. Soxhlet extraction

Ten grams of sample of each particle size and 200 mL of solvent were used for each extraction. They mixture was introduced into a soxhlet extractor for three hours, during which six extraction cycles took place. The solution obtained in the extraction process was distilled if the solvent was ethanol-water (80/20) and both components, the solid and liquid, were weighed and introduced in an oven for 24 h at a constant temperature of 100 ± 5 °C. Then, they were weighed again to study the differences in weight, and to obtain the data of the yield during the process.

When distilled water was used as solvent, the solution obtained in the extraction was introduced directly into the oven (100 ± 5 °C), producing the evaporation of the water and thus obtaining immediately the tannins.

2.3.2. Extraction with agitation and heat

In this procedure, 200 mL of the solvent solution and 10 g of sample were mixed and introduced into a Kytasato flask and then place over a magnetic stirrer with a hot plate (80 ± 5 °C) (model SBS. A-63) for a period of three hours. For reasons of efficiency, the Kytasato flask was covered with a cellulose stopper in order to avoid sample losses due to the agitation. As in the Soxhlet extraction, if the solvent was ethanol-
water (80/20) the solution obtained in the extraction process was distilled and both components, the solid and liquid, were weighed and introduced in an oven for 24 h at a constant temperature of 100 ± 5 °C. They were then weighed again to study the differences in weight, and to obtain the data of the yield during the process.

2.4. Chemical characterisation of extracts

The Folin-Ciocalteu method (Miranda et al., 2016; Singleton and Rossi, 1965), was used to determine the total phenol content, using a Beckman DU-70 spectrophotometer, and absorbance reading at 760 nm. A gallic acid calibration curve at five concentrations (50, 25, 10, 5, and 0 ppm) was used. Total phenol content was expressed as mg gallic acid/g dry bark extract.

Anthocyanins measurements were done by the acid hydrolysis in butanol method (Bikoro Bi Athomo, 2018). The absorbance was recorder at 530 nm and the results were expressed as cyanin equivalent based on dry bark extract. A cyanine calibration curve at five concentrations (30, 15, 10, 5, and 0 ppm) was used. A standard calibration method was followed, the commercial cyanine chloride standard, 1 mg, was dissolved in 1 mL of methanol and the 5 mL flask was made up to the mark with distilled water. From this solution, which is 200 ppm, the necessary dilutions are prepared to make the standard line. And they undergo the same procedure as the samples. Experiments were done with different particle sizes (<0.5, 1.0–0.5, >1.0), and the analyses were done for triplicate.

2.5. Extracts antioxidant activity

The antioxidant activity was determined by the method FRAP (ferric reducing antioxidant power). The FRAP assay shows the extract potential to reduce Fe(III) to Fe(II). The antioxidant activity can be measured spectrophotometrically by the change in the solution colour to blue resulting from the reduction in Fe(III)-2,4,6-tripyridyl-s-triazine (TPTZ) to Fe(II)-2,4,6-tripyridyl-s-triazine (TPTZ). For this, 180 μL of the extract solution was mixed with 540 μL of distilled water and 5.4 mL of FRAP solution (83.3 % 0.3 M acetate bufer, 8.3 % 10 mM TPTZ and 8.3 % 20 mM ferric chloride v/v) and allowed to react for 30 min at 37 °C, after absorbance was measured at 595 nm. The results were expressed as trolox equivalents (TE).

2.6. Statistical analysis

Differences between particle size and extraction methods were tested for significance for both species and extracts, using Kruskal-Wallis test. Statistical analysis was performed using RStudio Computer Program (RStudio version 3.6.3; RStudio PBC).

3. Results and discussion

3.1. Soxhlet extraction

Fig. 1 shows the results of the Soxhlet extractions for P. pinaster and A. dealbata bark samples, with different diameters and solvents. Particles with a diameter smaller than 0.5 mm and the use of ethanol as solvent produce the highest yields. A. dealbata highest yield is 29.5 wt% and P. pinaster highest yield is 25.8 wt%.

As results indicate, the particle size is a key point in the performance of the extraction process, the smaller the particle size, the highest the yield is. Both species obtained the highest yields with particle size <0.5 mm, and the lowest yields with particle size > 1 mm. Baldosano et al. (2015), confirmed that particle size has an effect on tannin extraction, finding that fine particles produce higher tannin yields, as they offer greater surface area for mass transfer (Baldosano et al., 2015). Al-Sumri et al. (2017), used different particle size rangers to study date extraction of oil for date stones using a soxhlet apparatus, and found the smallest particle size range gave the best results (Al-Sumri et al., 2017). This also agrees with Muhammad Muheyiddin et al. (2018), who showed that tannin extraction from the bark of Rhizophora mucronata gave highest yields with a 0.5 mm milled bark particle size than with bigger sizes (Muhammad Muheyiddin et al., 2018). These results are also confirmed by the Kruskal-Wallis tests performed. There were significant differences (p < 0.05) between particle sizes for both species which indicates the influence of this variable in the percentage of yields extracted.

In addition, the use of ethanol as a solvent provides a higher extraction performance if we compare it with the results obtained with distilled water. The best results for P. pinaster Soxhlet extraction yield when using ethanol (25.8 wt%) is nearly three times higher than the yields obtained using distilled water as a solvent (9.3 wt%). A. dealbata best results are also obtained using ethanol: Yields obtained are three times higher with ethanol than with water for particles’ size <0.5 mm (29.5 wt% with ethanol and 10.1 wt% with water) and 0.5–1 mm (27.5 wt% with ethanol and 7 wt% with water), and five times higher for particles’ size >1 mm (24.9 wt% with ethanol and 5.2 wt% with water). These results are in also in agreement with the Kruskal-Wallis tests performed, which indicate there are significant differences between ethanol and distilled water. Rodrigues et al. (2018), performed soxhlet extraction of eucalyptus leaves using different solvents and found that polar solvents such as ethanol and methanol gave the highest yields compared to non-polar ones (Rodrigues et al., 2018). This also agrees with Seabra et al. (2018), who studied the influence of various solvents in the extraction of pine bark tannins and found using ethanol was the most favourable additive (Seabra et al., 2018). Other studies such as the one from Singh et al. (2014), found that a combination of ethanol, diethy l ether and water (8:1:1) exhibited the highest yield content (Singh et al., 2014).

3.2. Extraction with agitation and heat

As stated previously in the methods section, those samples with larger particle size presented great difficulties during the experiments, leading to very large errors in the results. For this reason, once the samples were analysed the authors decided to discard the particles with size >0.5 mm.

Fig. 2 shows the yields obtained from P. pinaster and A. dealbata. The species with the highest yields is A. dealbata for both solvents (11.9 wt% for ethanol and 9.9 wt% for water). Yields obtained from A. dealbata using water as a solvent (9.9 wt%) is nearly three times higher than P. pinaster (3.6 wt%) using the same solvent. However, as in the soxhlet methods, the highest yields are achieved using ethanol as a solvent, being the yields obtained with this solvent very similar for both species (11.9 wt% for acacia and 10.5 wt% for P. pinaster). Kruskal-Wallis test shows there were significant differences between solvents for P. pinaster. The same test, however, shows no significant differences between solvents for A. dealbata using the agitation and heat method.

Nevertheless, the highest average yield obtained, which is by
A. dealbata, did not exceed 12 %. This is less than half the highest average yield obtained with the soxhlet extraction, also obtained by A. dealbata (29.5 wt%), indicating extraction by soxhlet is the most suitable methods at laboratory scale to maximize the total extraction yield. In addition, there were significant differences when comparing methods of extraction for each of the species. This is agreements with previous studies that have shown that in general a soxhlet extraction is more effective than other techniques. When comparing the soxhlet method with a boiling technique to extract tannins from Rhizophora barks, Muhammad Muhayyidin et al. (2018), showed that the soxhlet method was superior to boiling, obtaining 29 wt% and 13 wt% of extractives respectively (Muhammad Muhayyidin et al., 2018). Markom method with a boiling technique to extract tannins from Acacia dealbata (Muhammad Muhayyidin et al., 2018) also studied the effects of different extraction methods to obtain tannins from Phyllanthus niruri and showed extraction by soxhlet was the best method for gallic and ellagic acid extractions. Rodrigues et al. (2018), extracted tannins from Eucalyptus globulus by soxhlet extraction and by supercritical fluid extractions, obtaining the highest yields with the soxhlet extraction (Rodrigues et al., 2018).

3.3. Chemical characterization of the extracts

This opens the possibility of knowing phenolic and anthocyanin content by knowing the absorbance of the samples, by using methods such as Folin-Ciocalteu and acid/butanol. This is in concordance with other studies that used the same techniques to determine tannin composition of tree species (Antoine et al., 2004; Bikoro Bi Athlomo, 2018; Pelozo and Mello, 2008).

The chemical properties of the extracts obtained for both methods and solvents are shown in the Table 1.

For P. pinaster, phenol and anthocyanin content is higher for particle’s size < 0.5 mm for both methods. Kruskal-Wallis test showed significant differences between particle sizes for both compounds in both extraction methods. When comparing methods, phenol and anthocyanin content is also higher using the soxhlet method than the agitation and heat. There are significant differences between methods for phenol content, according to the tests performed. However, for anthocyanin content, no significant differences were found between methods.

For A. dealbata, phenol and anthocyanin content is higher for particle’s size 0.5–1 mm for both methods. Kruskal-Wallis test showed significant differences between particle sizes for phenol in both in both extraction methods, and for anthocyanin in the agitation and heat methods. However, Kruskal-Wallis showed no significant differences for anthocyanin in particle size when using the soxhlet method. When comparing methods, phenol and anthocyanin content obtained is higher with the agitation and heat method than using a soxhlet. Tests performed show no significant difference between extraction methods for phenol content. However, anthocyanin content between methods are significantly different.

Our results are on the same range as other studies. Rosales et al. (2009), showed that for different Pinus species, the concentration of total phenols ranged between 491 mg gallic acid/g dry sample to 604 mg gallic acid/g dry sample, being the concentrations higher in organic extracts than in crude extracts (Rosales-Castro et al., 2009). Furthermore, Razi et al. (2019), showed the optimal extraction process for each chemical component should be designed in order to improve polyphenols content and condensed tannins. They extracted tannins from barks of Acacia mellissima obtaining values of 481.4 mg GAE/g bark using a 80 % methanol solvent and 80.16 mg CYA/g bark using a 20.5 % methanol solvent (Rhazi et al., 2019). These results are also in the same range of other species such as Eucalyptus spp., studied by Lima et al. (2017). They analysed the total phenol content of 11 species of eucalyptus and results ranged from 283 to 917 mg/g of extract (Lima et al., 2017). On the other hand, similar studies agree that with a smaller particle size the results are better (Kopjar et al., 2015; Yildiz et al., 2018). Therefore, it highlights the great difference obtained in the phenolic and anthocyanin concentrations in the acacia samples in this study. This could be due to when collecting the samples, the protocol did not differentiate between young trees and mature trees. If we analyse the results of Chemetova et al. (2020), we can see that the differences obtained can be double depending on the age of the tree. In addition, the experiments done with the smallest particle size (<0.5 mm) were carried out without any difficulty. However, as the sample size increased, the stirrer began to malfunction producing constant stopages during the agitation process and an overheating of the samples. As a consequence of this, the results of these analyses could suffer some alteration due to these causes. Therefore, this parameter must to be analysed in the future researches.

This study shows that what is proposed here is a technically viable alternative that should be evaluated from and energy and consumption point of view to determine if it is profitable. In addition, management of resources, such as reduction of waste of the wood extracted from pines, and management of an invasive species such as acacia, following eradication plans, would be carried out.

3.4. Antioxidant activity

The ferric reducing antioxidant power (FRAP) of all the non-polar extracts to Acacia dealbata was 412 ± 10 TE/g and 386 ± 5 TE/g with ethanol and water, respectively. Pinus pinaster obtained a value to 145 ± 2 and 126 ± 3 for ethanol and water respectively. Ethanol was more efficient than water to extract compounds with antioxidant activity, with FRAP values between 7 and 15 % higher for ethanol when compared with the equivalent water extracts. Acacia dealbata bark extract showed the highest FRAP values with more than double those obtained by Pinus pinaster bark.

| Extraction Method | Species | Particle Size (mm) | Phenol content (mg gallic acid/g extract) | Anthocyanin (mg cyanin chloride/g extract) |
|-------------------|---------|------------------|----------------------------------------|--------------------------------------|
| Soxhlet           | Acacia dealbata | <0.5             | 232.5 ± 4.7                            | 91.3 ± 1.2                           |
|                   |          | 1–0.5            | 820.2 ± 9.5                            | 233.9 ± 7.0                           |
|                   |          | >1               | 250.4 ± 6.1                            | 77.3 ± 12.4                           |
|                   | Pinus pinaster | <0.5             | 873.2 ± 9.0                            | 552.4 ± 10.0                          |
|                   |          | 1–0.5            | 477.8 ± 15.1                           | 301.6 ± 15.2                          |
|                   |          | >1               | 675.0 ± 18.1                           | 426.9 ± 5.3                           |
| Heating and stirrer | Acacia dealbata | <0.5             | 661.1 ± 16.0                           | 218.4 ± 7.0                           |
|                   |          | 1–0.5            | 775.4 ± 8.6                            | 247.3 ± 3.6                           |
|                   | Pinus pinaster | <0.5             | 302.4 ± 3.9                            | 110.5 ± 4.8                           |
|                   |          | 1–0.5            | 524.9 ± 11.6                           | 459.2 ± 4.5                           |
|                   |          | >1               | 404.2 ± 10.5                           | 269.0 ± 37.2                          |
|                   |          | 379.6 ± 8.1      | 217.8 ± 8.1                            |
4. Conclusions

This paper shows the results on the extraction yields and chemical composition of barks of *Pinus pinaster* and *Acacia dealbata*. The highest yields obtained were 25.8 wt% for *P. pinaster*, and 29.5 wt% for *Acacia dealbata*. Ethanol (80-20 vol/vol) was the most efficient solvent agent compared to distilled water, providing better yields in both extraction methodologies. The particles with size smaller than 0.5 mm in diameter were the one that gave a better average of extraction provided by the different species and methods. Although these results are similar to those from other studies. A yield of less than 30 wt% could be improve different species and methods. Although these results are similar to were the one that gave a better average of extraction provided by the methodologies. The particles with size smaller than 0.5 mm in diameter. Ethanol (80-20 vol/vol) was the most efficient solvent agent yields obtained were 25.8 wt% for *A. dealbata*.

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