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The Effect of Different Solvents on Extraction Yield, Total Phenolic Content and Antioxidant Activity of Extracts from Pine Bark (*Pinus pinaster* subsp. *atlantica*)

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Pine bark extracts are a rich source of natural polyphenols with potential beneficial antioxidant properties. The extraction yield, antioxidant activity, as well the type of polyphenols recovered not only depend on the extraction method but also on the solvent used for extraction.
Thus, the aim of the present study was to ascertain the effect of different solvents on extraction yield, total phenolic content and antioxidant activity of extracts from *Pinus pinaster* subsp. *atlantica*, the major forest biomass of Northwest of Portugal.
Soxhlet extractions with water, water/ethanol (1/1) and just ethanol, as solvents, were performed for 4 hours.
Best results were obtained with the water/ethanol mixture. Overall, the solvent's performance was as follows: water/ethanol> ethanol> water.

1. Introduction

Pinus pinaster (maritime pine) can be found in some Western European countries, such as Portugal, Spain, France, and in some Northern African countries (Chupin et al., 2015). The Portuguese pine sector is a vital component of the total forest economic value (around 17 %), being the third most important species after eucalyptus and cork oak (Seabra et al., 2012). Pine bark is an abundant residue of the wood industry, since it represents 10–20% of the pine tree trunk. Considering the global trend of circular economy which includes recovering, recycling and upgrading wastes, pine bark can be used as a raw material. In contrast to grape pomace, a common residue generated by the Portuguese agricultural activities, pine bark is available all year long, and it is cheaper, more stable and easier to handle and process (Braga et al., 2008). Pine bark is a useful source of phenolic compounds, with procyanidin oligomers being the predominant phenolics. Typical phenolic compounds present in pine bark are (+)-catechin, (−)-epicatechin, dihydroquercetin, as well as phenolic acids.
In the past years, additives have been incorporated into foods to improve quality, however the toxicity of certain compounds and the recent search by the consumers of natural alternatives has increased the interest of the food industry to use compounds of natural origin (Oreopoulou and Tzia, 2007). In particular, great interest has been recently focused on the addition of polyphenols to foods and biological systems due to their well-known abilities to scavenge free radicals, i.e. antioxidant power (Pinelo et al., 2004, Seabra et al., 2012). Some studies have been published on the utilisation of pine bark phenolics in meat products (Ahn et al., 2004, Hameş-Kocabaş et al., 2008, Shah et al., 2014) and yogurt (Ruggeri et al., 2008).
Actually, already exists a patented product - Pycnogenol® (US Pat. No. 4,698,360) which is a polyphenol concentrate extracted from the maritime pine bark (*Pinus maritima L.* or *Pinus pinaster* Aiton, subsp. *atlantica*) from the Bay of Biscay in Gascogne, used throughout the world as a food supplement with a protective effect against chronic and degenerative diseases (D' Andrea, 2010).
Polyphenols is a big group of molecules characterized by having an aromatic ring and a hydroxyl group. These are compounds of the plant secondary metabolism that can accumulate in certain plant organs such as leaves, fruits, roots and stems. As a large group of bioactive chemicals, they have diverse biological functions.

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Because they are essential to plant life, they can provide defence against microbiological attacks and make food inedible to predators and other herbivores (Oliveira et al., 2014). These aromatic compounds are divided in alkali soluble phenolic compounds and alkali insoluble phenolic compounds. The former belongs to the flavonoid family. The latter has a complex structure that appears to be related with the lignin of wood (Vázquez et al., 1987). These compounds are soluble in methanol, hot water and ethyl acetate (Fengel and Wegener, 1984). In bark, the main fraction of polyphenols is composed by hydrolysable tannins and condensed tannins. The former are mixtures of simple phenols such as pyrogallol and ellagic acid, and esters of a sugar, mainly glucose, with gallic and digallic acids. Condensed tannins consist of flavonoid units (essentially flavan-3-ols and flavan-3,4-diols) which have suffered varying degrees of condensation, and are associated with carbohydrates and traces of amino and imino-acids (Fradinho et al., 2002). The bark of many conifers consists mainly of condensed tannins, also called proantocyanidins (Fengel and Wegener, 1984).
Most of flavones and flavonols are obtained in the dichloromethane fractions and ethyl acetate extract of pine because of their low polar nature. The presence of flavonoids and phenolic acids in various species of pine has been variously reported (Kahlouche-Riachi et al., 2015). To extract flavonoid glycosides and higher molecular weight phenolics, solvents of higher polarity like ethanol or ethanol–water mixtures can be used, resulting in higher yields of total extracted polyphenols. Due to such a huge group of aromatic phytochemicals, several solvents must be evaluated to yield the higher amount of molecules of interest and to decrease concomitantly the treatment costs (Ayala-Zavala et al., 2011). One study reported selective extraction of flavan-3-ol monomers, catechin and flavonols preferentially in the organic phase, whereas procyanidins were extracted in the aqueous phase (Bonilla et al., 1999). The proanthocyanins, oligomers of the catechin and epicatechin, are highly soluble in ethyl acetate (Thorat et al., 2013).
The aim of this work was to assess the effect of ethanol, 50 % aqueous ethanol and water as extraction solvents on extraction yield, total phenolic content and antioxidant activity of pine bark extracts.

2. Materials and methods

2.1 Pine Bark Sample Preparation

Pine bark (*Pinus pinaster* subsp. *atlantica*) was collected in Minho region, Northwest of Portugal, from trees aged 15 years. The bark was dried to reach equilibrium humidity at 40 °C for 72 hours. Further it was ground in a Termomix TM31 (Vorwerk, Germany) and sieved to select the particles between 200 and 850 µm. The bark was kept in sealed bags for further experiments.

2.2 Reagents

Food grade ethanol 96 % was purchased from Aga (Prior Velho, Portugal). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and gallic acid monohydrate were purchased from Sigma-Aldrich (Steinheim, Germany). Ascorbic acid and Folin-Ciocalteu reagent were purchased from Merck (Darmstact, Germany). Sodium carbonate anhydrous was purchased from Panreac (Barcelona, Spain). Methanol was purchased from Jt Baker (Deventer, Holland). The reagents were of analytical grade. Deionised water was used.

2.3 Soxhlet extraction

Classical Soxhlet equipment was used in this study. In summary, a total of 12.5 g of ground pine bark was put into an extraction thimble and placed inside the upper reservoir. Then, 220 mL of solvent (water or 50% aqueous ethanol or just ethanol) were added to the lower reservoir and the mixture boiled for 4 h under reflux. The solid/liquid ratio was 1:17.6 (w/v). The number of cycles and lengths were recorded. After cooling, the extract was filled up to 250 mL with the respective solvent (extract stock solution).

2.4 Extraction yield

The extraction yield (in % (w/w) - a measure of the solvent efficiency to extract specific components from the original material, defined as the amount of solid extract recovered in mass compared with the initial amount of dry bark (Aspé and Fernandez, 2011) was determined for each one of the solvents tested and dried at 103 °C.

2.5 Total phenolic content

Total phenolic content of pine bark was determined colourimetrically at 725 nm, using the method described by Lafka et al. (2007) based on Gutfinger (1981). Extract stock solution (0.5 mL) was mixed with 20 mL of deionised water in a 25 mL volumetric flask. Then, 0.625 mL of Folin-Ciocalteu reagent was added and mixed thoroughly. After 3 minutes, 2.5 mL of sodium carbonate was added and made up to final volume with deionised water. The absorbance was read after 30 minutes at 725 nm (Hitachi U-1100 double-beam UV/vis spectrophotometer). A mixture of water and reagents was used as a blank. The results were obtained as mg

of gallic acid equivalent (GAE) per g of dry bark. The standard curve was prepared with gallic acid in water at 0, 10, 25, 50, 75, 100, 125, 150, 175, 200, 250 and 300 mg/L.

2.6 Antioxidant activity

The antioxidant activity of the extracts was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method used by Deng et al. (2011) based on Brand-Williams et al. (1995). A volume of 2 mL of diluted extract stock solution (in methanol) was mixed with 6 mL of DPPH in methanol and allowed to stand at room temperature, in the dark, for 30 minutes prior to measuring the solution absorbance at 517 nm. The control was a DPPH solution containing absolute methanol instead of the sample. The antioxidant activity was based on the measurement of the reducing ability of pine extracts towards the radical DPPH. The results were obtained as mg of ascorbic acid equivalent (AAE) per g of dry bark. The standard curve was prepared with ascorbic acid at 0, 1, 2.5, 5, 7.5, 10, 20, 30, 35 and 40 mg/L.

2.7 Statistical analysis

The extractions and analysis were performed in triplicate and the data presented as mean ± SD values. SPSS (IBM, USA) was used for statistical analyses. The analysis of variance (ANOVA) and the Tukey test were used to determine statistically different values at a significance level of $p<0.05$.

3. Results and Discussion

3.1 Extraction yield

Figure 1 shows the solvent effect on extraction yield. The mixture water/ethanol and only ethanol showed no significant differences and have yielded the higher amount of extract (17.55 ± 0.16 % and 17.08 ± 0.23 %, respectively).

Do et al. (2014) studying phenol extraction from *Limnophila aromatica*, using various solvents, also observed that 50 % aqueous ethanol showed the higher extraction yield, however better results were obtained with 100 % water rather than 100 % ethanol. The antioxidant components are of a phenolic nature therefore, organic solvents of higher polarity are more effective in quantitative recovery of these substances than nonpolar solvents (Oreopoulou, 2003).

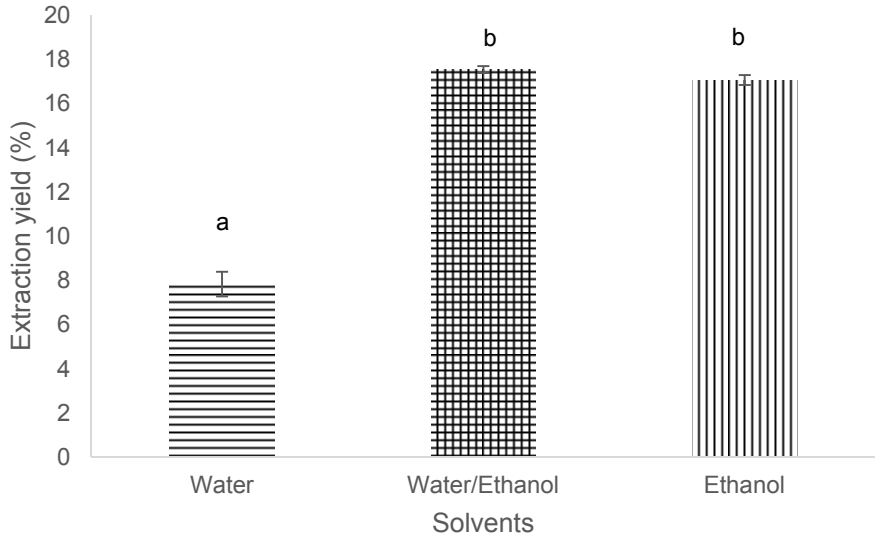

Figure 1: Extraction yield of pine bark extracted with different solvents. Means (n=3) with different lowercase letters in the same graph are significantly different (p≤0.05).

3.2 Total phenolic content (TPC)

Solvent type significantly affected the total phenolic content ($p<0.05$), and the mixture water/ethanol extracted the higher content of phenolic substances (73.48 ± 1.84 mg GAE/g DM), followed by ethanol and water (63.38 ± 1.26 mg GAE/g DM and 50.09 ± 4.70 mg GAE/g DM, respectively), as can be seen in Figure 2.

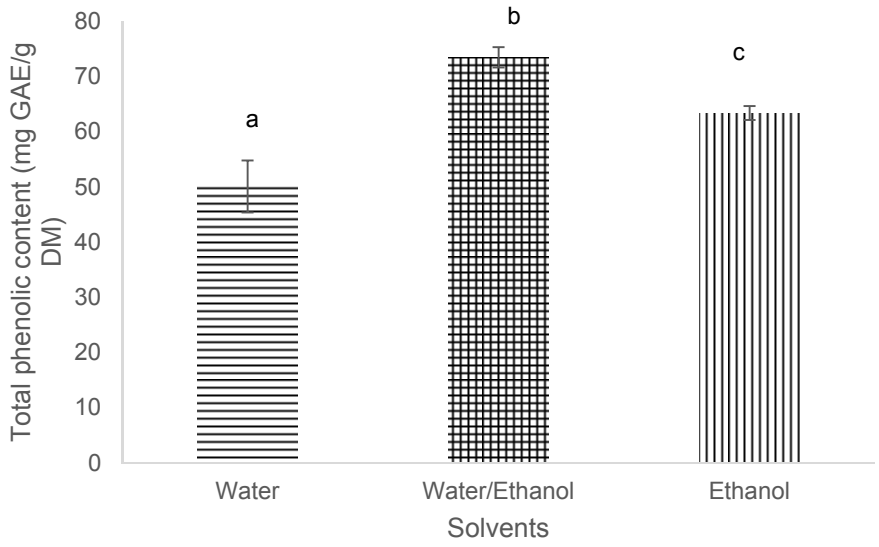

Figure 2: Total phenolic content of pine bark extracted with different solvents. Means (n=3) with different lowercase letters in the same graph are significantly different (p≤0.05).

Chupin et al. (2015) obtained 18.07 ± 3.82 mg GAE/g bark when extracting pine bark with 80 % aqueous ethanol by microwave assisted extraction (MAE). Aspé and Fernandez (2011) who studied the effect of different extraction techniques on total phenolic content of pine bark, observed a higher value for Soxhlet extraction when comparing with MAE, with one-stage extraction. Other study observed that a mixture of water/ethanol was a better solvent than pure water or pure ethanol for the extraction of phenolic compounds because it extracted more polyphenolic compounds (Bai et al., 2010). In addition, Pinelo et al. (2004) obtained better results when extracting pine sawdust with ethanol compared to water. The higher TPC obtained in this study could also be due to the use of a moderate ethanol concentration as referred by Bai et al. (2010).

3.3 Antioxidant activity

Water/ ethanol extracts showed the best antioxidant activity (108.74 ± 2.02 mg AAE/g DM), followed by ethanol and water extracts (95.58 ± 0.55 and 82.24 ± 4.65 mg AAE/g DM, respectively) (Figure 3).
Jusoh et al. (2017) stated that the highest DPPH of apple peel extract can be achieved when ethanol concentration is between 10 - 47 %, beyond that value, the DPPH value starts to decrease. The proposed explanation is that by increasing the ethanol content the polarity changes which, thus, reduces the tendency of the solvent to extract compounds that can react with DPPH.

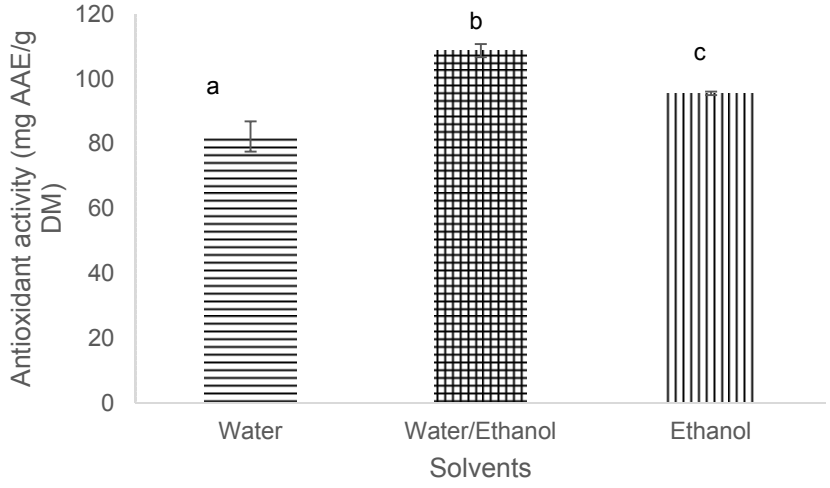

Figure 3: Antioxidant activity of pine bark extracted with different solvents. Means (n=3) with different lowercase letters in the same graph are significantly different (p≤0.05).

3.4 Correlation of TPC and antioxidant activity

Figure 4 shows a linear correlation of total phenolic content with DPPH scavenging ability in all extracts (R^2=0,983). Such high correlation value suggests that the antioxidant activity value (DPPH method) can be predicted based on TPC (Folin-Ciocalteu method). A high correlation confirms yet that the phenolic compounds present in the extracts are responsible for its antioxidant activity.

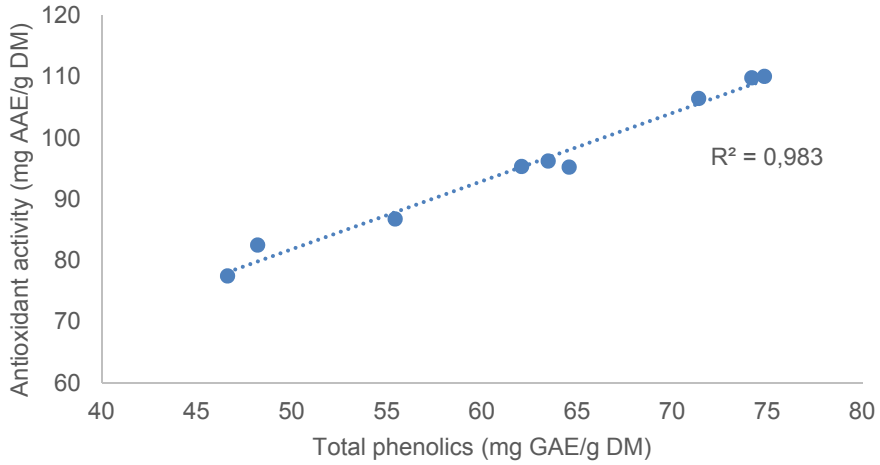

Figure 4: Relationship between total phenolic contents and DPPH (mg AAE/g DM).

4. Conclusions

Overall the best results were obtained with the mixture water/ethanol, followed by ethanol and water. The highest recovery of TPC with a value of 73.48 ± 1.84 mg GAE/g DM can be achieved by using a water/ethanol mixture (1/1). There is a linear and high relationship of TPC and antioxidant activity, showing that TPC is a good indicator of antioxidant activity.

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