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Inhibitory Effects of *Pinus Pinaster* Aiton Subsp.A*tlantica* Bark Extracts Against Known Food Pathogens

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Pine bark extracts are a rich source of natural polyphenols that besides their known antioxidant activity may also exhibit significant antimicrobial properties. In the current study, the antimicrobial activity of *Pinus pinaster* Aiton subsp. *atlantica* extracts was screened against gram-positive bacteria (*Bacillus cereus*, *Clostridium perfringens*, *Listeria monocytogenes*, *Staphylococcus aureus)*, gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica* serovar Enteritidis) and the fungus *Aspergillus brasiliensis* ATCC 16404, using the disk diffusion assay. The hydroethanolic extracts obtained by Microwave assisted extraction (MAE) at 1600 W and 110 ºC for 30 minutes, were compared to the Soxhlet extracts. The pine bark was dried to reach equilibrium humidity at 40 °C for 72 hours and milled to a particle's size of 200-850 µm. The extraction yield was determined by the amount of extract recovered in mass compared to the initial amount of dry bark and the total phenolic content was determined by the Folin Ciocalteu colorimetric method at 725 nm. Hydroethanolic pine bark extracts were prepared at 0.25, 0.50 and 80.00 mg/mL concentrations.
MAE attained higher extraction yield and higher total phenolic content (20.4±0.7% w/w and 71.1±3.4 mg of gallic acid equivalent (GAE) per g of sample, respectively) than Soxhlet extracts (17.6±0.2% w/w and 54.9±2.6 mg GAE/g sample, respectively).
The pine bark extracts presented inhibition halos ranging from 7 to 17 mm against the gram-positive bacteria, namely *C. perfringens* ATCC 13124 and *B. cereus* NCTC 11143. The MAE extracts presented higher inhibition halos than the Soxhlet ones. Regarding the tested concentrations, only *C. perfringens* ATCC 13124 and *B. cereus* NCTC 11143 were sensitive to the 0.25 and 0.50 mg/mL concentrations. At 80 mg/mL all the gram-positive bacteria were sensitive to the pine bark extracts obtained by both extraction methods. On the other hand, the extracts did not show inhibitory effect against the tested gram-negative bacteria and *A. brasiliensis.*This study reveals that MAE is a fast and efficient method for obtaining *Pinus pinaster* Aiton subsp. *atlantica* extracts with antimicrobial activities against Gram-positive bacteria.

1. Introduction

Pine wood is commonly used for producing pulp and board, but all bark is removed prior to the chipping process due to the high lignin/polyphenol content, which causes difficulties in processing. The bark removed from logs is mostly used as boiler fuel, but a huge surplus is still discarded as a waste residue. In fact, pine bark is an important biomass resource, amounting to about 10–15% of the total tree weight (Ku et al., 2007). The extraction of bark or heartwood of some forest species for phenolic substances, has been investigated for a long time (Jorge et al., 2002). In Portugal, the pine sector is a vital element of the total economic value of the forest, around 23% (Ferreira-Santos et al., 2019), being the third most important species after eucalyptus and cork oak (Seabra et al., 2012, Ferreira-Santos et al., 2019). Maritime pine (*Pinus pinaster* subsp. *atlantica*) is a conifer native to Mediterranean countries such as France, Spain and Portugal, as also in some North African countries (Chupin et al., 2015). There are two different commercially-available pine bark extracts from *P. pinaster*, namely Pycnogenol® and Flavangenol® (Mármol et al., 2019).

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Great interest is currently focused on potential benefits of the addition of bioactive compounds to food products, due to their known antioxidant and antimicrobial activities, these last arousing the scientific interest mainly due to the high number of microorganisms resistant to emerging antibiotics (Seabra et al., 2012, Balasundram et al., 2006, Chupin et al., 2015, Jerez et al., 2007).
Polyphenolic compounds are ubiquitous in all plant organs and are, therefore, an integral part of the human diet. There are 8000 phenolic structures reported which are widely dispersed throughout the plant kingdom, and many occur in food (Oroian and Escriche, 2015).
Infectious diseases caused by bacteria resistant to multiple drugs have become one of the most serious problems nowadays. Although the use of antibiotics has greatly reduced the incidence of infectious diseases, it has also led to the appearance of drug-resistant bacteria. Various plant phenolics, including phenolic acids, flavonoids and tannins known to be synthesized by plants in response to microbial infection, have been shown to possess a broad spectrum of antibacterial effects against a wide array of microorganisms and a number of effective drugs have been created against them (Ignat et al., 2013). Furthermore, it was shown that these compounds have low toxicity to the living systems (Tanase et al., 2018).
Some studies have been published on the utilisation of Pycnogenol in meat products (Ahn et al., 2004, Ahn et al., 2007, Hameş-Kocabaş, 2008) and yoghurt (Ruggeri et al., 2008).
Hameş-Kocabaş (2008) observed that the addition of 1% of Pycnogenol to meat reduced the growth of *Staphylococcus aureus* during storage when compared to control. Ahn et al. (2007) verified that Pycnogenol reduced the numbers of *Escherichia coli* O157:H7 and *Salmonella typhimurium* and retarded the growth of *Listeria monocytogenes* and *Aeromonas hydrophila*. Ruggeri et al. (2008) observed that the enrichment of yoghurt with Pycnogenol did not promote any fermentative activities of the lactic acid bacteria, nor did it affect the protein and lipid contents. This suggests the utilization of Pycnogenol as a valuable ingredient to enrich yogurt preparation.
Extraction of plant materials can be done by various extraction procedures. Many extraction methods have been used to obtain condensed tannins (Chupin et al., 2015). Classical techniques for the solvent extraction of nutraceuticals from plant matrices are based on the choice of solvent coupled with the use of agitation and/or heat. Traditional extraction methods include maceration, Soxhlet extraction, and percolation; however, they are often time-consuming, require relatively large quantities of solvents, and the active compounds sometimes degrade (Aspé and Fernández, 2011, Vieito et al., 2019). Non-conventional methods, are more environmental friendly due to decreased use of synthetic and organic chemicals, reduced operational time, and better yield and quality of extract (Azmir et al., 2013). Microwave assisted extraction (MAE) is a fast and efficient unconventional extraction method that generates a high pressure in the biomaterial that leads to a rupture of the cells, thus improving the penetration of the extracting solvent. The fact that it is fast, has high extraction rates, uses low amounts of solvent, and extracts both polar and non-polar compounds, makes it a promising extraction method (Chupin et al., 2015, Ferreira-Santos et al., 2019). This technique involves extraction with controlled pressure and temperature. The use of closed vessels shortens the extraction time and increases the extraction efficiency. This method has been applied for the extraction of phenolic compounds from plant material (Oroian and Escriche, 2015).
The present study compares the ability of traditional and new techniques to extract *P. pinaster* Aiton subsp. *atlantica* bark compounds. The antimicrobial activity of the extracts was screened against gram-positive bacteria (*Bacillus cereus*, *Clostridium perfringens*, *Listeria monocytogenes*, *Staphylococcus aureus*), gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica* serovar Enteritidis) and the fungus *Aspergillus brasiliensis*, using the disk diffusion assay.

2. Materials and methods

2.1 Pine Bark Sample Preparation

Pine bark (*P. pinaster* Aiton subsp. *atlantica*) was collected in Minho, a Northwest region of Portugal (Viana do Castelo, Portugal), from trees aged 15 years. The inner bark was separated from the outer bark and the latter cut into pieces, and oven dried to reach equilibrium humidity at 40 °C for 72 hours. The dried outer bark was ground by using a mixer (Termomix TM31, Vorwerk, Germany) for 20 s and sieved at an amplitude of 0.2 for 1 min to select the particles with a diameter between 200 and 850 μm. All analyses and extractions were performed on outer dried pine bark.

2.2 Reagents

Food grade ethanol 96% was purchased from Aga (Prior Velho, Portugal). Gallic acid monohydrate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Steinheim, Germany). 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). Bleach was purchased from Neoblanc (Fater SpA, Italy). The reagents were of analytical grade.

2.3 Preparation of pine bark extracts

2.3.1 Soxhlet extraction

The extraction was performed according to the methodology described by Vieito et al. (2018) using classical Soxhlet equipment and 50% aqueous ethanol as solvent.

2.3.2 Microwave assisted extraction

The microwave assisted extraction with SK-12 medium pressure rotor (ETHOS X, Milestone, Italy) was used. The irradiation occurred at a microwave power of 1600 W, at 110 °C for 30 minutes. A total of 2.5 g of pine bark was placed in the extraction vessel and 50 mL of solvent (50% aqueous ethanol) were added. After the extraction, four vessels were mixed together and completed to 200 mL.

2.4 Extraction yield

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

2.5 Total phenolic content

Total phenolic content (TPC) of pine bark was determined colorimetrically at 725 nm, using the method described by Singleton and Rossi (1965). The extract (0.5 mL) was mixed with 0.5 mL of Folin-Ciocalteu reagent. After 3 minutes, 0.5 mL of sodium carbonate was added and made up to final volume (10 mL) with deionised water. The absorbance was read after 90 minutes at 725 nm (VWR® Spectrophotometers, UV-Vis Scanning UV-3100PC). 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, 5, 10, 20, 40, 60, 80, 100, 120 and 160 mg/L.

2.6 Antimicrobial activity

For this assay, the extract was lyophilized for 48 hours, under vacuum, in an Alpha 1-2 LDplus freeze-dryer (Christ, Germany) and dissolved in DMSO. Both, Soxhlet and Microwave extracts were assayed for antimicrobial activity at the concentration 0.25, 0.50 and 80.00 mg/mL. These concentrations (0.25 and 0.50 mg/mL) were chosen based on toxicity assays on human intestinal CaCo-2 and HaCat keratinocytes cells (data not shown).

A disk diffusion assay was used to determine the diameter of the inhibition zone of tested extracts and was performed following the method by CLSI (2012). Each disk (Oxoid, England) (6 mm in diameter) was impregnated with 10 µL of extract or control (DMSO and bleach coded as Lx). Strains of *Aspergillus brasiliensis* ATCC 16404, *Bacillus cereus* NCTC 11143 and ATCC 11778, *Clostridium perfringens* ATCC 13124, *Escherichia coli* ATCC 25922 and ATCC 8739, *Listeria monocytogenes* ATCC 13932, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and ATCC 29213, and *Salmonella enterica* serovar Enteritidis ATCC 25928 were inoculated in Columbia Agar + 5% Sheep Blood (COS, Biomérieux, France). Active cultures (0.5 McFarland) were spread with a cotton swab onto Mueller-Hinton Agar (MHA, Oxoid, England). In the case of *A. brasiliensis*, MHA was supplemented with 0.2% glucose as described in Lacasa et al. (2007). Plates were allowed to dry for 3 to 5 minutes. Flame sterilized tweezers were used to place the disks onto inoculated MHA plates. The plates were allowed to stand for 15 minutes and then they were inverted and incubated for 22±2 h at 37±1 °C. Zones of inhibition were measured in mm with the help of ImageJ software (Rasband, 1997-2018). The values presented correspond to the mean of the two inhibition halos. DMSO was used as negative control.

2.7 Statistical analysis

The chemical analyses 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

The extraction yield of antioxidant compounds from plant material is influenced mainly by the conditions under which the process of liquid–solid extraction is carried out (Oroian and Escriche, 2015).
Figure 1 shows the performance of the extraction method on extraction yield. Significant differences were found between the two methods (p<0.05). MAE exhibited a higher amount of extract, 20.4±0.7% (w/w) than Soxhlet extracts, 17.6±0.2% (w/w). Aspé and Fernández (2011) observed that with only one stage of extraction, the Soxhlet technique performed better than MAE, however with multiple stages of extraction, concluded that MAE yielded the most.

3.2 Total phenolic content

The type of extraction technique significantly influenced the TPC (Figure 1, p<0.05). The extraction techniques in this parameter, showed similar behaviour to the extraction yield, with MAE attaining higher TPC (71.1±3.4 mg GAE/g sample) than Soxhlet (54.9±2.6 mg GAE/g sample). The TPC value was lower with Soxhlet probably due to the length of time that the sample was exposed to high temperatures (boiling temperature of solvent).

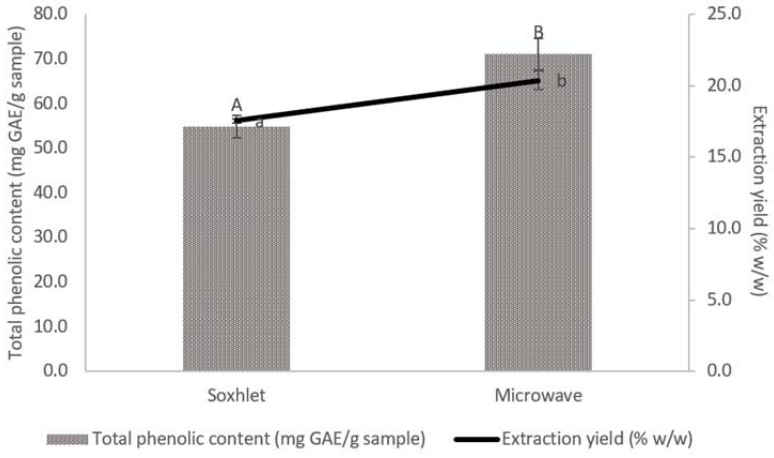

Figure 1: Extraction yield and Total phenolic content of pine bark extracted. Means (n=3) with different superscript letters in the same graph are significantly different at p<0.05 according to the Tukey test.

3.3 Antimicrobial activity

The pine bark extracts presented inhibition halos ranging from 7 to 17 mm against the gram-positive bacteria, as can be seen in Table 2. The MAE extracts presented higher inhibition halos than the Soxhlet ones. Regarding the tested concentrations, only *C. perfringens* ATCC 13124 and *B. cereus* NCTC 11143 were sensitive to the 0.25 and 0.50 mg/mL concentrations. At 80.00 g/mL all the gram-positive bacteria were sensitive to the pine bark extracts obtained by both extraction methods. According Kumar and Brooks (2017), gram-positive bacteria have been found more susceptible than gram-negative bacteria due to the latter having an outer double-layer membrane, highly hydrophilic lipopolysaccharide molecules and a unique periplasmic space. On the other hand, the extracts did not show inhibitory effect against the tested gram-negative bacteria and *A. brasiliensis*.

Table 1. Antimicrobial activity of the hydroethanolic extracts of P. pinaster Aiton subsp. atlantica bark determined by the disk diffusion method (inhibition halo measurements in mm).

		Soxhlet concentration extracts (mg/mL)			Microwave Concentration extracts (mg/mL)		
Microorganism	**Reference**	**0.25**	**0.50**	**80.00**	**0.25**	**0.50**	**80.00**
A. brasiliensis	ATCC 16404	–	–	–	–	–	–
B. cereus	NCTC 11143	7.8±0.2	7.6±0.0	11.0±0.3	–	8.4±0.1	13.0±0.1
	ATCC 11778	–	–	10.6±0.5	–	–	12.3±0.1
C. perfringens	ATCC 13124	7.9±0.4	8.2±0.4	15.8±0.3	7.8±0.4	8.2±0.2	17.1±0.0
E. coli	ATCC 25922	–	–	–	–	–	–
	ATCC 8739	–	–	–	–	–	–
L. monocytogenes	ATCC 13932	–	–	9.9±1.6	–	–	11.4±0.3
P. aeruginosa	ATCC 27853	–	–	–	–	–	–
S. aureus	ATCC 25923	–	–	10.3±1.4	–	–	13.5±0.1
	ATCC 29213	–	–	9.0±0.2	–	–	11.5±0.3
Salmonella enterica	ATCC 25928	–	–	–	–	–	–

Legend: "–"no inhibition halo "0.0±0.0"

4. Conclusions

Microwave extraction (MAE) was the technique that most favoured extraction, both in the extraction yield and total phenolic content. In addition, it was faster and more efficient than Soxhlet.

Pinus pinaster Aiton subsp. *atlantica* extracts exhibited antimicrobial activity against the tested gram-positive bacteria, with the MAE extracts presenting the best inhibition halos.

The obtained results highlight the great potential of MAE pine bark extracts to be used on biotechnological processes due to its antimicrobial activity against some Gram+ bacteria, such as *Clostridium perfringens* and *Bacillus cereus*, even at very low concentrations.

In conclusion, instead of being disposed as waste or utilized in low value applications, pine forest-residues, such as bark, being promising sources of important constituents showing biological properties, should be considered for valorisation.

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