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Influence of Extraction Solvent on the Biological Properties of Maritime Pine Bark (*Pinus pinaster*)

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Abstract

Maritime pine bark (*Pinus pinaster* Aiton subsp. *atlantica*) is rich in polyphenols with known bioactive properties which are beneficial for human health. However, biological activities of bark extracts depend on the type of polyphenols extracted and the characteristics of these extractives depend on several factors such as the type of solvents used. The influence of the extraction solvent on the composition and consequently on the properties of the extracts has been poorly described. Thus, in this study the influence of the extraction solvent (water, ethanol and ethanol-water (50/50 v/v%)) on the antibacterial and anticancer properties of *P. pinaster* bark samples were evaluated. LC-DAD-MS profiling of the different extracts was also carried out to study their polyphenol composition. Results show that extraction solvent must be carefully chosen with respect to foreseeing use of bark extracts, since ethanolic and hydroethanolic extracts displayed the greatest antibacterial activity whereas water extracts showed increased anticancer properties.

Keywords: *Pinus pinaster*; Pine bark; Extraction solvent; LC-DAD-MS; Antibacterial; Anticancer

1 Introduction

Plants have been used worldwide for traditional medicine remedies due to their antioxidant, anti-inflammatory and antimicrobial properties (Elgazar et al., 2019; Ginovyan et al., 2017; Toiu et al., 2019). Nowadays, plant-derived metabolites are of great interest as alternatives to the current treatments of a wide range of diseases, including cancer, metabolic syndrome and neurodegenerative disorders (Taghipour et al., 2019; Zhao et al., 2019). Among all plant metabolites, polyphenols are highlighted due to their health-promoting properties that include antioxidant, anti-inflammatory, immunomodulatory, chemoprotective and anti-diabetic activities (Ganesan & Xu, 2017; Gorzynik-Debicka et al., 2018; Sánchez-González et al., 2017). It remains un-

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clear whether single molecules are responsible for the observed effects or it is the result of a synergy between two or more compounds, since evidence supporting both hypotheses have been obtained (Gascón et al., 2018; Kristek et al., 2019; Lewandowska et al., 2014).

Bioactive compounds are present in edible and non-edible parts of plants, but the use of edible parts for medicinal purposes raises an ethical issue due to a growing global hunger crisis. For this reason, non-edible parts such as bark or peel have been studied as new sources of molecules with promising biological activities (Amri et al., 2017; Mármol et al., 2019).

Pine bark is an abundant by-product of the pulp, paper and sawmill, and building industry, in which trees are felled with different ages/trunk diameters according to their use. Pine bark has been widely investigated due to its high content of polyphenols, thus repurposing a by-product from forest processing as a source of bioactive compounds. Of all the pine species, maritime pine (*Pinus pinaster* Aiton subsp. *atlantica*) has been one of the most investigated with respect to its health-promoting effects (Mármol et al., 2019). Three maritime pine bark extracts are commercially available as dietary supplements, all claiming that the pine originates from Les Landes de Gascogne forest in southwestern France: Pycnogenol®, (Horphag Research, Switzerland), Olygopin®, (DRT, France) and Flavangenol®, (Toyo Shinyaku Inc., Japan). Pycnogenol®, is by far the most studied extract of *P. pinaster* with proven health-promoting effects (Feragalli et al., 2018; Oliff & Blumenthal, 2019; Pourmasoumi et al., 2020).

Some authors have reported the influence of different extraction methods on the biological properties of plant-derived fractions. Final composition of the extracts might be heavily influenced by extraction procedure, which in turn modifies their biological activities as well as other properties such as taste (Mzid et al., 2017; Rodrigues et al., 2015; Xu et al., 2018). Since the loss of a bioactive compound and/or a group of them might invalidate the therapeutic potential of plant extracts, all extraction steps must be carefully planned according to the intended purpose.

There is a growing concern to use greener solvents for extraction, such as water and ethanol, in order to reduce the environmental impact.

Herein, we analyse the composition and biological properties of samples from *Pinus pinaster* Aiton subsp. *atlantica* bark from the Minho region, Northwest of Portugal, extracted with three different solvents: ethanol, water, and ethanol-water (50/50 v/v%), in order to evaluate the influence of the extraction solvent on antimicrobial and anticancer activities.

2 Materials and Methods

2.1 Obtention of pine bark extracts

Maritime pine bark (*Pinus pinaster* Aiton subsp. *atlantica*) was collected in the Minho region, Northwest of Portugal, from trees aged 15 years. Whole bark was manually separated into an inner layer (phloem) and outer layer (rhytidome) based on morphological and colour differences between bark layers. Only the outer layer was used in this experiment. Bark samples were cut into smaller pieces, oven dried at 40 ^{o}C for 72 h, ground (Termomix TM31, Vorwerk, Germany) and sieved at the amplitude of 0.2 mm for 1 min to select the particles of 200 to 800 μm diameter. Bark samples were subjected to Soxhlet liquid-solid extraction using deionized water (PW), ethanol (PE) and water-ethanol (50/50 v/v%) (PWE) as solvents, as described in previous experiments (Vieito et al., 2018). 12.5 g of dry ground pine bark was put into an extraction thimble and placed inside the upper reservoir. Then, 220 mL of each solvent was added to the lower reservoir and the mixture boiled for 4 h under reflux. After cooling, the extract was filled up to 250 mL with the respective solvent (extract stock solution). Water and ethanol were selected as environmentally safe and food grade solvents. These liquid extracts were used in the subsequent analyses.

2.2 Analysis of pine bark extracts' composition by liquid chromatography-photodiode-array-mass spectrometry

Liquid chromatography-photodiode-array-mass spectrometry (LC-PDA-MS-MS) data was obtained with a Vanquish liquid chromatography system (Thermo Fisher Scientific®,) coupled to Ultimate 3000 UV Detector (Dionex®,) and Q Exactive Plus mass spectrometer (Thermo Fisher Scientific®,). LC conditions were as follows: Luna C-18 column (150 x 3 mm i.d., 3μm, Phenomenex®, Torrance, USA); solvent system, (A) H_2O containing 0.1% formic acid, (B) MeCN with 0.1% formic acid; gradient mode for aqueous pine bark extracts (PW): 5% of B for 1 min, 5 to 50% of B for 15 min, 50 to 100% of B for 5 min, and 100% of B for 3 min; gradient mode for ethanolic (PE) and hydroethanolic (PWE) extracts: 5% of B for 1 min, 5 to 100% of B for 19 min, and 100% of B for 3 min; flow rate at 500 μL/min; injection volume was 10 μL; sample concentration was 5 mg dry extract/mL H_2O/MeOH. UV detection was performed at 254 and 280 nm. ESI-MS conditions were as follows: collision energy 35 eV; capillary temperature 320^{o}C; electrospray negative ion mode (source voltage: 2500 V) in full scan and ms2, mass range: 100-1500.

2.3 Determination of antibacterial activity of pine bark extracts

15 mL of the extract stock solution were lyophilized for 48 h, under vacuum, in an Alpha 1-2 LDplus freeze-dryer (Christ, Germany) and reconstituted in 2 mL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). DMSO, used here as a "negative control", has a broad dissolving capacity both polar and non-polar compounds, and is relatively inert and widely used in biological assays. The final concentration of the applied extracts was 30 mg extract/mL DMSO for PW and 65 mg extract/mL DMSO for extraction using PWE and PE. They were reconstituted in DMSO equivalent to the extraction yield obtained in the Soxhlet extraction. The extraction yield (defined as the amount of solid extract recovered in mass compared with the initial amount of dry bark) of water (PW) extracts (7.84 ± 0.56%) was significantly lower compared to ethanolic (PE) and hydroethanolic (PWE) extracts respectively, 17.55 ± 0.16 % and 17.08 ± 0.23 % (Vieito et al., 2018). Hence, it was decided to use the same volume of extract that reflects the different mass corresponding to the yield of each respective solvent. The extracts with antibacterial properties will be applied directly, just after reconstitution in water, as well as a food flavouring onto a salami-like product.
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 CITA. Each disk (Oxoid, England) (6 mm in diameter) was impregnated with 10 μL of extract or control (two disks per extract in a total of six disks per plate and two disks for the controls – DMSO and commercial solution of sodium hypochlorite coded as Lx (Neoblanc, Fater SpA, Italy)). Strains of *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). Plates were allowed to dry for 3 to 5 min. The disks were placed onto inoculated MHA plates, one disk in each of the eight equal parts. The plates stood for 15 min and then were inverted and incubated for 22 h ± 2 h at 37 ^{o}C ± 1 ^{o} 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 and bleach were used, respectively, as negative and positive controls.
Bacterial strains were selected as the most representative of the gram-positive and gram-negative foodborne pathogenic and food spoilage bacteria.

2.4 Cell lines

Three types of tumoral human cell lines were tested: colorectal adenocarcinoma Caco-2 cells, breast adenocarcinoma MCF-7 cells and hepatocellular carcinoma HepG2 cells. The cell lines were provided by different sources which are further acknowledged. All cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37^{o}C. Cells (passages 20-40) were grown in Dulbecco's Modified Eagles medium (DMEM) (Gibco Invitrogen, Paisley, UK) supplemented with 20% fetal bovine serum, 1% non-essential amino acids, 1% penicillin (1000 U/mL), 1% streptomycin (1000 μg/mL) and 1% amphotericin (250 U/mL). Culture medium was replaced every two days and cells were passaged enzymatically with 0.25% trypsin-1 mM EDTA and sub-cultured on 25 cm^2 flasks at a density of $2{\cdot}10^4$ cells/cm^2.
Experiments in undifferentiated Caco-2 cells as well as on MCF-7 and HepG2 cells were performed 24 h post-seeding. For assays on differentiated Caco-2 cells, cells were cultured on 96-wells plates under standard culture conditions for 7 to 9 days, until reaching 80% confluence as confirmed by optic microscopy observation.

2.5 Cell viability assays

Cells were seeded in 96-well plates at a density of 4·103 cells/well. The culture medium was replaced with fresh medium (without foetal bovine serum) containing pine bark samples at concentrations varying from 0 to 1000 mg/L (for PE samples) or from 0 to 125 mg/mL (for PW and PWE samples), with an exposure time of 72 h. Thereafter, cell growth was analysed by the sulforhodamine B assay as previously described (Jiménez et al., 2016). Absorbance was measured with a scanning multiwell spectrophotometer (Biotex Sinergy ht Siafrtd, Vermont, USA) at wavelength between 540 and 620 nm. The effect on cell growth was expressed as a percentage of the control and calculated as % control. Experiments were conducted in quadruplicate wells and repeated at least two times. Results were expressed as mean ± SD.

3 Results and Discussion

3.1 Extraction solvent influences composition of pine bark extracts

Figures 1, 2 and 3 show, respectively, chromatographic data (LC-PDA-MS-MS) obtained for aqueous (PW), hydroethanolic (PWE) and ethanolic (PE) pine bark extracts. Major identified compounds in *Pinus pinaster* bark extracts using HPLC-DAD-MS analysis with electrospray negative ionization (ESI-) are numbered and listed in Tables 1 (aqueous extract) and 2 (hydroethanolic and ethanolic extracts, analysed under the same analytical conditions). The major polyphenolic compounds identified in the three types of *Pinus pinaster* bark extracts were catechin and taxifolin, two flavonoids of great interest due to their health-promoting properties (Li et al., 2020; Shafabakhsh et al., 2020; Sunil & Xu, 2019). Catechin, epicatechin and taxifolin represent "monomeric procyanidins" of which catechin was the most common (Oliff & Blumenthal, 2019). However, as it can be observed (Tables 1 and 2), PWE and PE extracts contained hydroxybenzoic acid, procyanidin, caffeic acid, abietic acid derivate and 15-hydroxydehydroabietic acid in their composition, whereas PW did not. On the other hand, the amount of taxifolin was greater in PW extracts than in PE and PWE extracts. Previous studies of Vieito et al. (2018) on pine bark extracts reported the higher antioxidant activity of hydroethanolic (PWE) extracts compared to water (PW) or ethanolic (PE) extracts. The dietary supplement Pycnogenol®, extracted from maritime pine bark using an ethanol-water solvent mixture (70/30 v/v%), showed significant antioxidant activity, based primarily on its procyanidin content (about 75%) (Oliff & Blumenthal, 2019).

3.2 PE and PWE extracts display greater antibacterial activity than PW extracts

The measurements of the inhibition halos of PW, PWE and PE extracts against the tested bac-

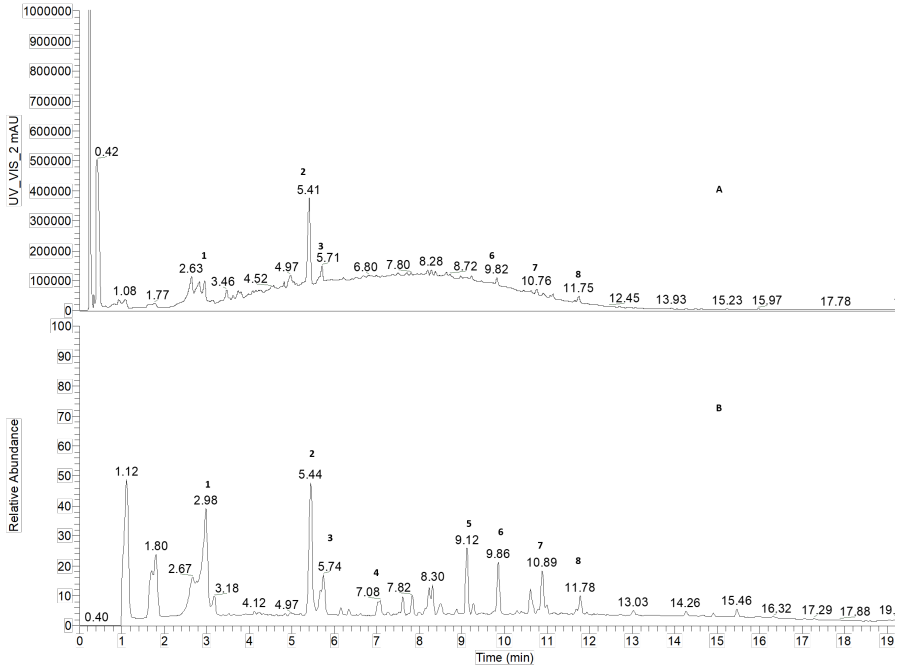

Figure 1: Chromatographic (LC-PDA-MS-MS) data obtained for aqueous *Pinus pinaster* bark extract (PW). A) HPLC profiles detected at 280 nm; B) ESI base peak ion chromatogram.

Table 1: Tentative structural elucidation of several chemical constituents contained in *Pinus pinaster* bark aqueous (PW) extract using HPLC-DAD-MS analysis with electrospray negative ionization (ESI-).

Peak[a]	Retention time (min)	λ_{max} (nm)[b]	m/z ESI- (MS2)	M	Area	Tentative identification	Reference
1	2.9	280	289.071	290	243 487	Catechin	a,b
2	5.4	290	303.050	304	1 158 143	Taxifolin 1	a,b
3	5.7	290	303.051 (285.040)	304	247 348	Taxifolin 2	a,b
4	7.0	290	505.192	-	11 192	ND	-
5	9.1	290	291.160	-	17 662	ND	-
6	9.8	286	289.144 (245.154)	-	66 895	ND	-
7	10.8	286	349.201, 331.191	-	49 871	Dehydroxydehydro abietic acid derivate	-
8	11.7	298	331.191	332	60 964	7,15-dihydroxydehydro abietic acid	c,d

[a] Peak numbering are shown in Fig. 1
[b] λ_{max} in UV spectrum from the PDA detector
ND- Not determined
References: a= Yesil-Celiktas et al. (2009), b= Almeida et al. (2016); c= Lee et al. (2018); d= Mulholland et al. (2017)

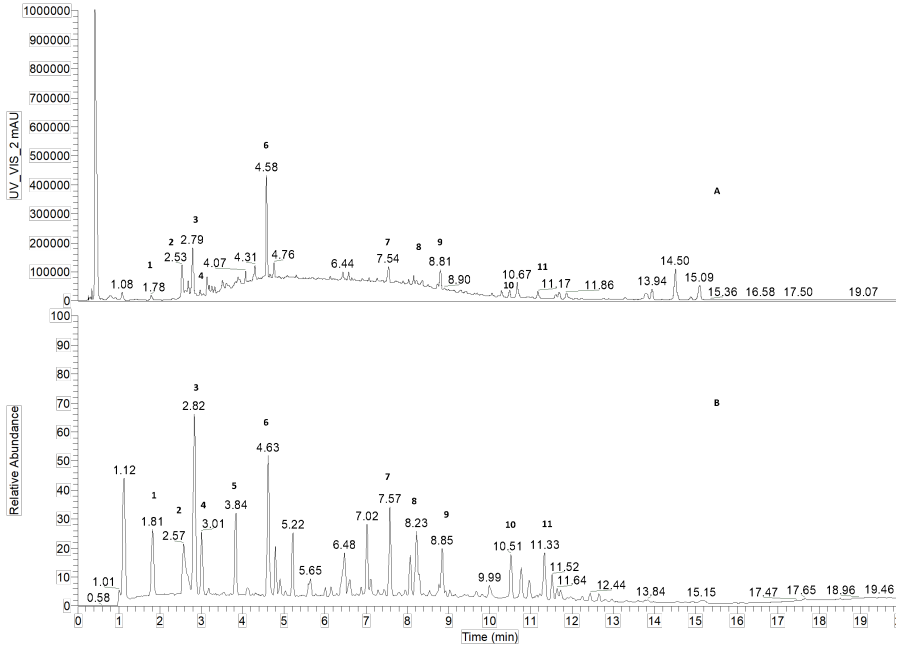

Figure 2: Chromatographic (LC-PDA-MS-MS) data obtained for hydroethanolic *Pinus pinaster* bark extract (PWE). A) HPLC profiles detected at 280 nm; B) ESI base peak ion chromatogram.

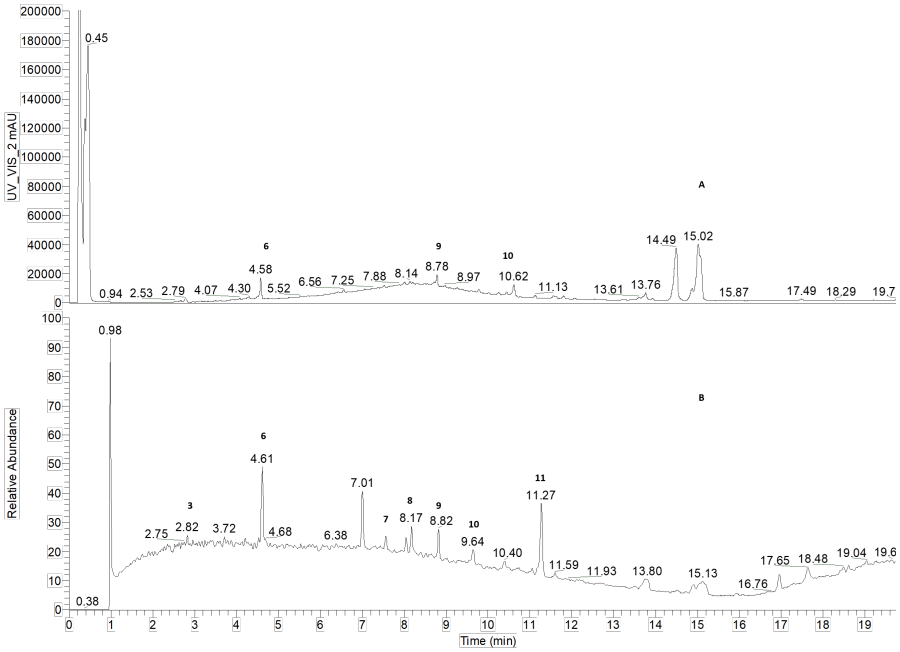

Figure 3: Chromatographic (LC-PDA-MS-MS) data obtained for ethanolic *Pinus pinaster* bark extract (PE). A) HPLC profiles detected at 280 nm; B) ESI base peak ion chromatogram.

Table 2: Tentative structural elucidation of several chemical constituents contained in *Pinus pinaster* bark hydroethanolic (PWE) and ethanolic (PE) extracts using HPLC-DAD-MS analysis with electrospray negative ionization (ESI-).

Peak[a]	Retention time (min)	λ_{max} (nm)[b]	m/z ESI-(MS2)	M	Tentative identification	Area		Reference
						PWE	PE	
1	1.8	229, 280, 308	137.023	138	Hydroxybenzoic acid	66 526	-	a
2	2.5	280	577.134	578	Procyanidin	256 425	7 402	b
3	2.8	279	289.071 (245.081)	290	Catechin	403 653	-	b, c, d
4	3.0	244, 323	179.034 (135.044)	180	Caffeic acid	38 250	-	c, d
5	3.7	263	186.112 (125.096)	-	ND	22 405	-	-
6	4.6	290	303.050	304	Taxifolin	715 433	44 845	c, d
7	7.5	263	289.144	-	ND	144 568	5 115	-
8	8.1	245, 303	275.165 (233.118)	-	ND	56 859	4 033	-
9	8.8	299	331.191	332	7,15-dihydroxydehydroabietic acid	148 233	17 046	e, f
10	10.5	284	333.207	-	Abietic acid derivate	68 784	27 323	-
11	11.2	276	315.196	316	15-hydroxydehydro-abietic acid	69 512	5 223	f

[a] Peak numbering are shown in Fig. 2 and 3
[b] λ_{max} in UV spectrum from the PDA detector
ND- Not determined
References: a= Touriño et al. (2005); b= Yesil-Celiktas et al. (2009); c= Almeida et al. (2016); d= Celhay (2013); e= Lee et al. (2018); f= Mulholland et al. (2017)

Table 3: Antibacterial activity of aqueous (PW), hydroethanolic (PWE) and ethanolic (PE) maritime pine bark extracts determined by the disk diffusion method in MHA (inhibition halos measurements).

Bacterial species	Strain reference	PW[a]	PWE[a]	PE[a]
B. cereus	NCTC 11143	8.4±0.0	11.2±0.2	10.7±0.3
	ATCC 11778	8.4±0.4	10.6±0.1	10.6±0.3
C. perfringens	ATCC 13124	11.9±0.7	15.6±0.3	14.4±0.3
E. coli	ATCC 8739	0.00±0.00	0.00±0.00	0.00±0.00
	ATCC 25922	0.00±0.00	0.00±0.00	0.00±0.00
L. monocytogenes	ATCC 13932	0.00±0.00	8.5±0.0	8.4±0.1
P. aeruginosa	ATCC 27853	0.00±0.00	0.00±0.00	0.00±0.00
S. aureus	ATCC 25923	9.2±0.5	11.5±0.1	11.4±0.2
	ATCC 29213	7.7±0.0	8.8±0.1	8.3±0.1
Salmonella Enteritidis	ATCC 25928	0.00±0.00	0.00±0.00	0.00±0.00

[a] Inhibition halos measurements in mm

Table 4: IC50 values (μg/mL) of *Pinus pinaster* bark ethanolic (PE), hydroethanolic (PWE) and aqueous (PW) extracts after 72 h of incubation on each cell line.

Pine bark extracts	Caco-2 cells IC50 values (μg/mL)	MCF-7 cells IC50 values (μg/mL)	HepG2 cells IC50 values (μg/mL)
PE	204.55±22.89	587.77±93.61	465.99±86.21
PWE	40.62±8.63	136.24±23.32	8.54±3.92
PW	15.06±9.37	53.23±9.14	2.36±0.73

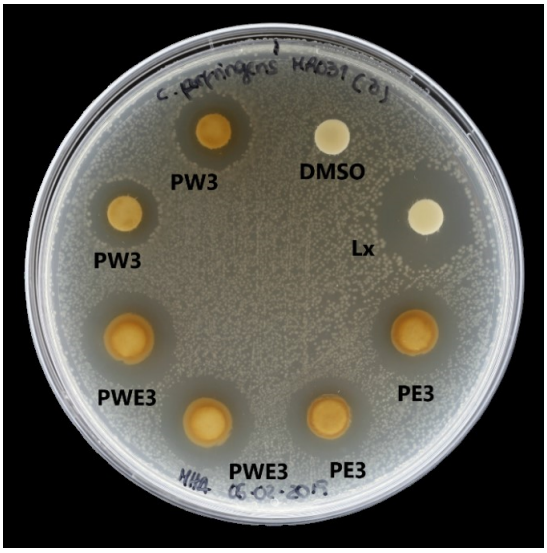

Figure 4: Antibacterial activity of maritime pine bark aqueous (PW), ethanolic (PE) and hydroethanolic (PWE) extracts against *Clostridium perfringens* ATCC 13124 by disk diffusion assay in MHA.

terial strains are presented in Table 3. All extracts presented bacterial activity against gram-positive bacteria, namely *B. cereus* NCTC 11143 and ATCC 11778, *L. monocytogenes* ATCC 13932, *S. aureus* ATCC 25923 and ATCC 29213, with the exception of PW which did not show antibacterial activity against *L. monocytogenes* ATCC 13932. The highest inhibition found was against *C. perfringens* ATCC 13124 whose inhibition halos can be observed in Figure 4. The extracts did not show antibacterial activity against the tested gram negative bacteria, namely *E. coli* ATCC 25922 and ATCC 8739, *P. aeruginosa* ATCC 27853 and *Salmonella* Enteritidis ATCC 25928. These results are in agreement with other studies that reported gram-positive bacteria to be more susceptible to antimicrobials than gram negative bacteria (Kumar & Brooks, 2018; Ramos et al., 2016). Activity of antimicrobials may be limited against gram-negative bacteria due to the outer double-layer membrane, highly hydrophilic lipopolysaccharide molecules and a unique periplasmic space (Kumar & Brooks, 2018). It also seems that hydroethanolic (PWE) and ethanolic (PE) solvents are better at extracting antibacterial compounds. As pointed out before, PWE and PE extracts contained hydroxybenzoic acid, procyanidin, caffeic acid, abietic acid derivate and 15-hydroxydehydroabietic acid. These polyphenolic compounds, known to have antioxidant properties and not found in PW extracts, may also have antimicrobial properties, and thus may be responsible for the inhibitory action against *L. monocytogenes* ATCC 13932. However, the same polyphenol may be effective on one type of gram-positive (or gram-negative) strain and ineffective on another type so indicating a strain-dependent effect (Bouarab-Chibane et al., 2019). Nevertheless, the antibacterial activity of maritime pine bark extracts against gram-positive bacteria can be a useful tool for food preservation purposes and, furthermore, a potential complement to antibiotic therapies in the treatment of bacterial infections.

3.3 PW extracts display the highest antiproliferative effect

The antiproliferative activity of *P. pinaster* bark extracts was analysed using a panel of selected

tumour cell lines, namely Caco-2 (human colorectal adenocarcinoma), MCF-7 (human breast adenocarcinoma) and HepG2 (human hepatocellular carcinoma). The obtained IC50 values are summarized in Table 4. Although all samples were able to significantly reduce cell viability, water (PW) extracts displayed the highest antiproliferative effect against all the analysed cell models, followed by water:ethanol (PWE) extracts and finally ethanol (PE) extracts, which could be due to the higher content of taxifolin in PW extracts. In terms of effectivity, HepG2 cells showed a better response to both PW and PWE samples followed by Caco-2 and finally MCF-7 cells, whereas Caco-2 cell line displayed a higher sensitivity to PE extracts than HepG2 and MCF-7.

In order to determine the selectivity of the extracts on healthy tissue, the cytotoxicity of all samples was evaluated on differentiated Caco-2 cells. This cell line undergoes spontaneous differentiation after reaching confluence, thus has been used as an intestinal barrier model to evaluate, for example, the absorption of nutrients and/or drugs. Differentiated Caco-2 cells displayed intracellular tight junctions, the characteristic brush border of healthy enterocytes and some of the most relevant enzymes of this cell type (Sambuy et al., 2005). In this line, all *P. pinaster* bark extracts were incubated 72 h on differentiated Caco-2 at different concentrations and IC50 values higher than 100 μg/mL were obtained for each extract, which suggested that samples might display tumour-selectivity.

The differences between the three extracts tested, in terms of antiproliferative effect, might be a consequence of the variations in their chemical composition as a result of the selected extraction solvent. As shown in Table 1, PW extracts contain a greater amount of taxifolin than PWE and PE extracts (Tables 1 and 2), which is a molecule of great interest due to its antitumoral activity. As reported by Sunil and Xu (2019), taxifolin presents several promising pharmacological activities of which anticancer activity is more prominent than other activities evaluated in either *in vitro* or *in vivo* models.

4 Conclusions

The influence of three extraction solvents, water, ethanol and ethanol-water (50/50 v/v%), on the chemical composition and biological activities of maritime pine bark (*P. pinaster* Aiton subsp. atlantica) was analysed. It was observed that the extraction solvent strongly determines the antibacterial and anticancer effectivity of the samples which might be due to differences in phenolic composition. The choice of an extraction solvent is dependent on the desired biological effect and, consequently, determines its potential applications.

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