Study of different extraction methods of bioactive molecules from different tree species

Leyre Sillero[a], Amaia Morales[a], Rut Fernández-Marín[a], Fabio Hernández-Ramos[a], Izaskun Davila[a], Xabier Erdocia[b], Jalel Labidi[a,*]

[a]Chemical and Environmental Engineering Department. University of the Basque Country UPV/EHU. Plaza Europa, 1, 20018 San Sebastian, Spain.
[b]Department of Applied Mathematics. University of the Basque Country UPV/EHU. Rafael Moreno "Pichichi", 3, 48013 Bilbao, Spain.
jalel.labidi@ehu.eus

The depletion of fossil resources and the growing environmental concerns of the actual society have leaded to an urgent need of shifting from petroleum-based products to natural substitutes. In this context, lignocellulosic biomass has emerged as a potential alternative to obtain bio-precursors for the synthesis of new chemicals and materials. Wood residues from unexploited Atlantic mixed forests have become an interesting type of biomass, not only for their content on structural compounds, but also for their non-structural fractions such as extractives. These compounds usually present antioxidant and antifungal capacities and, thus, they can be interesting for multiple applications. However, their extraction can be complex and costly and, hence, intensification techniques such as ultrasound assisted experiments have been studied. The aim of this work was the evaluation of the potential of five hardwood species coming from Atlantic mixed forests for the obtaining of biologically active compounds. For this aim, after studying the chemical composition of the woods, extractive compounds were obtained through ethanol-water based conventional and ultrasound assisted extraction methods. The obtained extracts were characterised and their antioxidant and antifungal capacities were determined. The yields of the extracts for ultrasound assisted experiments were, in general, higher than those for conventional ones and they also resulted to be richer in phenolic compounds. The obtained extractives seemed to have both antioxidant and antifungal activities, although the latest should be studied in depth in future works

1. Introduction

In recent years, mainly due to the depletion of fossil resources and the growing environmental concerns of the actual society, the research on natural substitutes for petroleum-based products has increased. For this purpose, the employment of different biomasses has been studied. The great interest in the use of biomass arises from its renewability and from its elevated availability, since it can be found in different areas, from forests to oceans. The positive contribution to the reduction of emissions of greenhouse gases is another important benefit generated by this feedstock, due to its theoretical zero CO_2 balance (Bonechi et al., 2017). Among all the different biomasses, the lignocellulosic biomass is the most promising one. Therefore, its use for the obtaining of added-value compounds is under development.

Lignocellulosic biomass is a three-dimensional polymeric composite material constituted mainly by cellulose, hemicelluloses and lignin, which are considered as structural compounds in combination with other minor non-structural compounds (pectins, inorganic compounds, proteins and extractives) (Octave and Thomas, 2009). The composition of this feedstock varies depending on the species, age, harvesting season and growing conditions (Hassan et al., 2018), which makes the standardisation of the valorisation process particularly complex.

One of the most studied lignocellulosic biomass is wood due to its high cellulose and lignin content. Nevertheless, apart from the structural compounds, it also has other added-value compounds that are present in smaller proportions, such as extractives. For the optimal valorisation of this biomass, an integral valorisation should be carried out, including every structural and non-structural fraction, which would lead to the compliance of the principles of circular economy and the zero waste strategy. For this aim, biorefinery processes have emerged in the last years. However, the recalcitrant nature of lignocellulosic biomass as well as its heterogeneity turns its conversion into a critical challenge (Hassan, 2018). Therefore, the selection of the most suitable fractionation methods is necessary in order to make the process economically profitable.

The extractive fraction in plants is a mixture of a multiple natural chemical products, including flavonoids, phenols, lignans, fatty acids, resins and terpenes, among others (Dou et al., 2016). Usually these molecules are secondary metabolites and some of them are bioactive. They are not part of the plant's structure, but are necessary for its survival, as they are essential for the defence mechanism against external agents (Azmir et al., 2013). Consequently, they have specific properties that are very useful for their application in different fields from bio-based materials to chemicals. Recent studies have shown that these compounds have different capacities as antioxidants and antifungals, among others. Synthetic preservatives (e.g. BHT, BHA, etc.) are widely employed, but there is an increasing concern about their negative effects on both people and the environment. Therefore, in the last years the demand of non-toxic natural preservative products has increased (Altemimi et al., 2017).

The extraction of natural products from plants has been conducted since ancient times. Currently, there are many different extraction methods, but the most widely used ones are solid-liquid extractions. They are considered conventional methods, and are based on the extraction of compounds according to their polarity. Some of the main disadvantages of these extraction techniques are the high-solvent consumption, the long extraction time, the need to use subsequent purification techniques and the possibility of degrading some of the target compounds (Luo et al., 2017). Therefore, in order to improve these limitations, in the last decades different intensification processes have been studied, where microwave and ultrasound are the most popular ones. Ultrasound assisted extraction (UAE) is based on the acoustic cavitation phenomenon, which favours the cell disruption, facilitating the mass transfer and the penetration (Vinatoru et al., 2017). Thus, an improvement in the extraction yield is achieved, with less solvent consumption, together with a reduction in energy and time consumption (Azmir, 2013).

Due to the decreasing forest harvesting in the Basque Country in the last few years, different species of hardwood trees have proliferated constituting the so-called Atlantic mixed forests. Black alder, black locust, common ash, common oak, Iberian with birch and sweet chestnut are some of the most common species of this type of Atlantic mixed forest (Sillero et al., 2020a). However, due to the slow growth of these species their commercial exploitation is not interesting even when some of them have an attractive market. Therefore, in order to carry out a sustainable management of the forests, it is essential to search for intermediate activities that could make a profit during the growth of the trees.

Based on this background, the aim of this work was the evaluation of the potential of different hardwood species for obtaining biologically active compounds. For that, firstly, the differences in the chemical composition of the woods were studied. Later, extractions were carried out with $EtOH/H_2O$ using conventional and ultrasound assisted extraction methods, and the obtained extracts were characterised and their antioxidant and antifungal capacities were determined.

2. Materials and methods

2.1 Raw material and chemicals

Wood samples from local forests in the Basque Country were collected and supplied by Basoekin Ltd. in the summer of 2020. The selected wood species were 5: *Betula pubescens, var celtiberica* (Iberian white birch), *Castanea sativa* (sweet chestnut), *Fraxinus excelsior* (common ash), *Quercus robur* (common oak) and *Robinia pseudoacacia* (black locust). These samples were debarked and grounded (Retsch Cutting Mill SM 100) to a particle size of 0.5 x 0.5 mm.

Scharlau supplied ethanol absolute, gallic acid, potato dextrose agar, sodium carbonate and Folin-Ciocalteu's phenol reagent. Sigma-Aldrich delivered 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) and Trolox. Toluene and methanol were obtained from Fisher Scientific. PanReac AppliChem supplied dimethyl sulfoxide (DMSO).

2.2 Chemical characterisation of wood

The five woods of different species were chemically characterised. Moisture, ash and extractive content were determined according to the standard methods (TAPPI T264-om-88, TAPPI T244-om-93 and TAPPI T204-cm-97, respectively) developed by the Technical Association of the Pulp and Paper Industry (TAPPI). The glucan, hemicelluloses and lignin content was analysed following the protocol NREL/TP-510-42618 of the National

Renewable Energy Laboratory (NREL). The quantification of the monosaccharides generated during the determination of the glucan and hemicelluloses was carried out by HPLC following the method described by Davila et al. (Dávila et al., 2017).

2.3 Extraction techniques

Two different extraction methods were compared to determine whether the intensification of the extraction with ultrasound has benefits in extracting bioactive compounds. In both extraction methods, $EtOH/H_2O$ (50/50 (v/v)) was used as solvent and a solid-liquid ratio of 1:10 (w/v). The conventional extraction (CE) method was carried out using an orbital shaker with temperature control (Heidolph Unimax 1010 with Heidolph Incubator 1000), and the working conditions were those previously optimised by Sillero et al. (2020b). Regarding the ultrasound assisted extraction (UAE), it was conducted in an ultrasound bath with temperature control (Elmasonic 570 H, Elma). In this case, the conditions used were those previously described by Sillero et al. (2019). The yield of the extractions were calculated gravimetrically and referenced to 100 g of dry wood. All measurements were carried out in triplicate.

2.4 Characterisation of the extracts

The obtained extracts were characterised to determine their total phenolic content (TPC), and their antioxidant and antifungal capacities were subsequently measured. The TPC determination was performed by Folin-Ciocalteu method (Singleton and Rossi Jr., 1965), using gallic acid as standard. The obtained results were reported as mg of gallic acid equivalents (GAE)/g of dried extract.

The antioxidant capacity of the extracts was evaluated using the DPPH method, which measures the quality of the hydrogen donors. Trolox was used as standard, and the methodology used for the measurements was that described by Sillero et al. (2018).

Trametes versicolor fungus was used to characterise the antifungal properties of the extracts. It was cultured on potato dextrose agar (PDA) at 25.5 ± 1.5 ºC for 7 days. To determine this capacity, first 10 mL of each of the obtained extracts were dried at 105 ºC for 1 day, and then resuspended in 1 mL of DMSO. 40 µL of the resuspended extracts were placed on a PDA-filled Petri dish, and then the dish was inoculated with a fungal strain with a fungal concentration of 9.16 x 10^6 spores/mL. Subsequently, these Petri dishes were sealed and incubated for 7 days at 25.5 ± 1.5 ºC in a climatic chamber (Selecta Medilow climatic chamber, JP Selecta S.A.). After this time, the growth intensity (GI) was determined by visual evaluation using the scale defined by ISO 846 and it is shown in Table 1. The samples were measured in triplicate.

Table 1: Visual assessment according to ISO 846.

GI	Evaluation
0	No growth apparent under magnification
1	No visible growth but visible under magnification
2	Visible growth up to 25 % coverage
3	Visible growth up to 50 % coverage
4	Visible growth up to 75 % coverage
5	Heavy growth covering more than 75 % of the studied area

3. Results and discussion

3.1 Different wood chemical composition

The difference in chemical composition between the woods of different species was demonstrated in this work (see Table 2). The glucan content of all the studied woods was similar, between 28-34 wt.%. Regarding the hemicelluloses content, it was noted that except for the Iberian white birch, all values were similar and in the range 19 to 24 wt.%. The hemicellulosic content of this wood was superior to 30 wt.%. Nevertheless, all the values measured in this work were in the hardwoods average range (Saidur et al., 2011). Iberian white birch was the wood with the lowest acid insoluble lignin content, below 20 wt.%. This was in accordance with what Sillero et al. (2020a) reported for the same raw materials. On the other hand, the Iberian white birch was the specie that demonstrated the highest amount of acid soluble lignin, above 5 wt.%, while the rest of the samples did not exceed 4 wt.%. Only the sweet chestnut had an ash content close to 3 wt.%, while the values for the other raw materials were lower or close to 1 wt.%, all of which can be considered typical values for hardwoods (Abdolali et al., 2014). The extractive content for all woods was in the range of 4.4 to 6.9 wt.%, confirming the influence of the studied species on the extractive content. Comparing the values calculated in this work with those reported by Sillero et al. (2020a) for the same raw materials (2-7 wt.%), it was seen that the values for this fraction were different, which confirmed the variability of this fraction depending on the growth conditions.

Table 2: Chemical characterisation (wt.%) of the wood of the five hardwood species

	Iberian white birch	Sweet chestnut	Black locust	Common ash	Common oak
Ash	0.48 ± 0.05	2.83 ± 0.09	0.70 ± 0.06	1.13 ± 0.04	1.10 ± 0.08
Extractives	4.49 ± 0.14	6.88 ± 1.15	4.49 ± 0.24	5.17 ± 0.47	6.32 ± 0.43
AIL	18.60 ± 1.66	24.43 ± 0.62	20.14 ± 0.85	22.25 ± 0.17	24.06 ± 1.14
ASL	5.48 ± 0.11	2.98 ± 0.27	2.62 ± 0.23	3.83 ± 0.26	3.39 ± 0.29
Glucan	32.03 ± 0.23	31.58 ± 0.91	32.34 ± 0.65	33.83 ± 0.15	28.56 ± 0.31
Hemicelluloses	30.55 ± 0.41	19.84 ± 0.08	18.95 ± 0.95	21.70 ± 1.04	23.96 ± 1.03

AIL: acid insoluble lignin; ASL: acid soluble lignin

3.2 Efficiency of extraction methods

Regarding the efficiency of the extraction processes, in general no significant differences were found between the use of the conventional extraction (CE) and the use of the ultrasound assisted extraction (UAE), as it is shown in Figure 1. Nevertheless, in the case of extractions from common ash wood, the extraction yield when the intensified extraction was performed was almost double. UAE allowed obtaining better or equal extraction yields in all cases except for Iberian white birch, where the use of ultrasound slightly decreased the extraction yield.
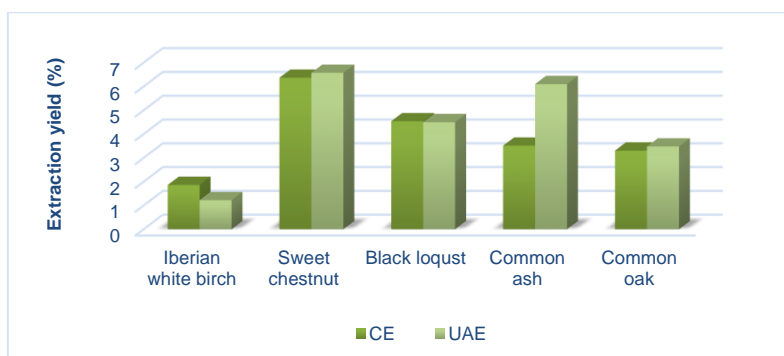

Figure 1: Extraction yields of the different extraction methods for the selected raw materials.

In the case of Iberian white birch and common oak woods, neither of the two types of extractions carried out resulted in the extraction of all the extracts that had been measured in their chemical composition. For common oak, the extraction of the 50 % of the total extractive content was achieved, while for Iberian white birch this percentage was less than 50 %. This could be due to the inaccurate choice of solvent or other reaction parameters. Thus, the need of optimising the extractions for the different woods is confirmed.

Comparing the extraction methods, in general it can be said that the UAE was better due to the fact that it improved the extraction yield together with the reduction of the extraction time and the energy consumption of the process. However, it would be important to optimise the extraction process for each of the woods in order to obtain the best extraction yield and avoid the degradation of the compounds.

3.3 Characterisation of the wood extracts

All the obtained extracts showed a significant phenolic compound content, with black locust wood extracts being the ones with the lowest TPC (see Table 3). Black locust wood extractions had yields of around 4 %, which is quite high, so the lower TPC may be due to the fact that this raw material is not rich in this type of compounds. These extracts also showed the lowest antioxidant capacity. This makes sense, as the antioxidant capacity is usually directly related to the phenolic compounds (Kainama et al., 2020). This work also confirms that the higher the TPC, the higher the DPPH.

The extracts of Iberian white birch were the ones with the highest phenolic compound concentration, as well as those with the highest antioxidant capacity, together with the extracts of sweet chestnut. Therefore, it can be concluded that the extraction yield does not ensure a high TPC, since the Iberian white birch extracts were the ones with the lowest yield, but they were the richest in phenolic compounds. Considering the results presented in Table 3, it can be said that the use of UAE improves the amount of extracted phenolic compounds except for sweet chestnut and common ash. This improvement in the extraction may be due to the cavitation phenomenon generated in the UAE, which favours cell disruption and facilitates the extraction of these compounds. The

common ash extractions carried out by UAE showed a better extraction yield, but the measured values of TPC and DPPH were worse than those obtained with CE. Probably, this was caused by the degradation of the samples as a result of extremely severe cavitation, or excessive exposure time. The TPC results measured in this work were lower than those measured by other authors for extracts from eucalyptus wood (Santos et al., 2013), as well as from Belgium apple wood (Withouck et al., 2019). The values of TPC and DPPH calculated in this work for wood extracts are far from those determined for the bark extracts of the same raw material (Sillero et al., 2019).

Table 3: Characterisation of the wood extracts.

	Extraction method	Iberian white birch	Sweet chestnut	Black locust	Common ash	Common oak
TPC (mg of GAE/g of dried extract)	CE	474 ± 30	391 ± 8	142 ± 17	284 ± 13	302 ± 22
	UAE	664 ± 28	361 ± 29	158 ± 12	209 ± 9	344 ± 32
DPPH (mg TE/g dried extract)	CE	394 ± 28	494 ± 10	54 ± 8	128 ± 13	228 ± 14
	UAE	495 ± 20	472 ± 32	71 ± 6	89 ± 4	273 ± 18

GAE: Gallic acid equivalent; TE: Trolox equivalent

The antifungal activity of the extracts was analysed in order to determine the ability of the extracted compounds to prevent fungal growth. Figure 2 shows the results obtained from the exposure of *Trametes versicolor* fungus to the different obtained wood extracts. In the case of both sweet chestnut and common ash, the growth intensity measured is 5 since the area where the drop was added is completely covered by the fungus. Looking at the results of the other three woods extracts, a small inhibition of the growth of the fungus is observed in all cases (GI = 4), being the Iberian white birch extract obtained by UAE the one that showed the lowest inhibition percentage. Both for Iberian white birch and black locust, the extracts obtained by CE presented slightly higher inhibition than those obtained by UAE. While in the case of common oak, the highest inhibition was detected with the extracts obtained by UAE. Based on the low inhibition results that have been measured, a higher concentration of the extracts is considered necessary to make the antifungal effect visible.
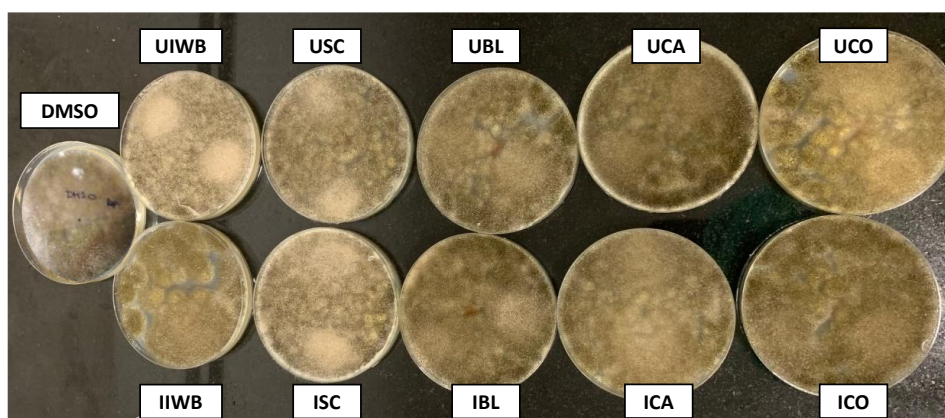

Figure 2: Antifungal activity measured for each of the extracts. UIWB: UAE Iberian birch bark; USC: UAE sweet chestnut; UBL: UAE black locust; UCA: UAE common ash; UCO: UAE common oak; IIWB:CE Iberian birch bark; ISC: CE sweet chestnut; IBL: CE black locust; ICA: CE common ash; ICO: CE common oak.

4. Conclusions

The characterisation of five hardwoods was satisfactorily performed, where the differences in the chemical compositions of the woods according to their tree species was confirmed.
The extractions were also successfully carried out, with significant extraction yields in all cases except for Iberian white birch, which remained low. The ultrasound assisted extractions showed, in general, a better capacity of extraction of phenolic compounds, with better extraction yields and higher TPC values than conventional extractions. It also shows one of the greatest inhibition capacities of the fungus growth, even though the concentration of extracts used was low. The Iberian white birch extract obtained by UAE was the most promising extract.

In conclusion, the intensification of the extraction by ultrasound favours the extraction of phenolic compounds with antioxidant and antifungal capacities. As a future work, the optimisation of the ultrasound assisted extraction for Iberian white birch wood is pending, in order to get the best profit from this raw material.

Acknowledgments

References

Abdolali A., Guo W.S., Ngo H.H., Chen S.S., Nguyen N.C., Tung K.L., 2014, Typical lignocellulosic wastes and by-products for biosorption process in water and wastewater treatment: A critical review, Bioresource Technology, 160, 57–66.

Altemimi A., Lakhssassi N., Baharlouei A., Watson D., Lightfoot D., 2017, Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts, Plants, 6(4), 42.

Azmir J., Zaidul I.S.M., Rahman M.M., Sharif K.M., Mohamed A., Sahena F., Jahurul M.H.A., Ghafoor K., Norulaini N.A.N., Omar A.K.M., 2013, Techniques for extraction of bioactive compounds from plant materials: A review, Journal of Food Engineering, 117(4), 426–436.

Bonechi C., Consumi M., Donati A., Leone G., Magnani A., Tamasi G., Rossi C., 2017, Biomass: An overview, Chapter In: F Dalena, A Basile, C Rossi (Ed.), Bioenergy Systems for the Future, Woodhead Publishing Limited, Cambridge, UK, 3–42.

Dávila I., Gullón P., Andrés M.A., Labidi J., 2017, Coproduction of lignin and glucose from vine shoots by eco-friendly strategies : Toward the development of an integrated biorefinery, Bioresource Technology, 244, 328–337.

Dou J., Galvis, L., Holopainen-Mantila U., Reza M., Tamminen T., Vuorinen, T., 2016, Morphology and Overall Chemical Characterization of Willow (Salix sp.) Inner Bark and Wood: Toward Controlled Deconstruction of Willow Biomass, ACS Sustainable Chemistry & Engineering, 4(7), 3871–3876.

Hassan S.S., Williams G.A., Jaiswal A.K., 2018, Emerging technologies for the pretreatment of lignocellulosic biomass, Bioresource Technology, 262, 310–318.

Kainama H., Fatmawati S., Santoso M., Papilaya P.M., Ersam T., 2020, The Relationship of Free Radical Scavenging and Total Phenolic and Flavonoid Contents of Garcinia lasoar PAM, Pharmaceutical Chemistry Journal, 53(12), 1151–1157.

Luo Y., Wu W., Chen D., Lin Y., Ma Y., Chen C., Zhao S., 2017, Optimization of simultaneous microwave/ultrasonic-assisted extraction of phenolic compounds from walnut flour using response surface methodology, Pharmaceutical Biology, 55(1), 1999–2004.

Octave S., Thomas D., 2009, Biorefinery: Toward an industrial metabolism, Biochimie, 91(6), 659–664.

Saidur R., Abdelaziz E.A., Demirbas A., Hossain M.S., Mekhilef S., 2011, A review on biomass as a fuel for boilers, Renewable and Sustainable Energy Reviews, 15(5), 2262–2289.

Santos S.A.O., Vilela C., Freire C.S.R., Neto C.P., Silvestre A.J.D., 2013, Ultra-high performance liquid chromatography coupled to mass spectrometry applied to the identification of valuable phenolic compounds from Eucalyptus wood, Journal of Chromatography B, 938, 65–74.

Sillero L., Barriga S., Izaguirre N., Labidi J., Robles E., 2020a, Fractionation of non-timber wood from Atlantic mixed forest into high-value lignocellulosic materials, Journal of Wood Chemistry and Technology, 40(3), 200–212.

Sillero L., Prado R., Andrés M.A., Labidi J., 2019, Characterisation of bark of six species from mixed Atlantic forest, Industrial Crops and Products, 137, 276–284.

Sillero L., Prado R., Labidi J., 2018, Optimization of Different Extraction Methods to Obtaining Bioactive Compounds from Larix Decidua Bark, Chemical Engineering Transactions, 70, 1369–1374.

Sillero L., Prado R., Welton T., Labidi J., 2020b, Energetic and environmental analysis of different techniques for biomolecules extractions, Chemical Engineering Transactions, 81, 631-636.

Singleton V.L., Rossi Jr. J.A., 1965, Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents, American Journal of Enology and Viticulture, 16(3), 144–158.

Vinatoru M., Mason T.J., Calinescu I., 2017, Ultrasonically assisted extraction (UAE) and microwave assisted extraction (MAE) of functional compounds from plant materials, TrAC Trends in Analytical Chemistry, 97, 159–178.

Withouck H., Boeykens A., Vanden Broucke M., Moreira M.M., Delerue-Matos C., De Cooman L., 2019, Evaluation of the impact of pre-treatment and extraction conditions on the polyphenolic profile and antioxidant activity of Belgium apple wood, European Food Research and Technology, 245(11), 2565–2578.