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Simultaneous Microwave-Ultrasound Assisted Extraction of Bioactive Compounds from Bark

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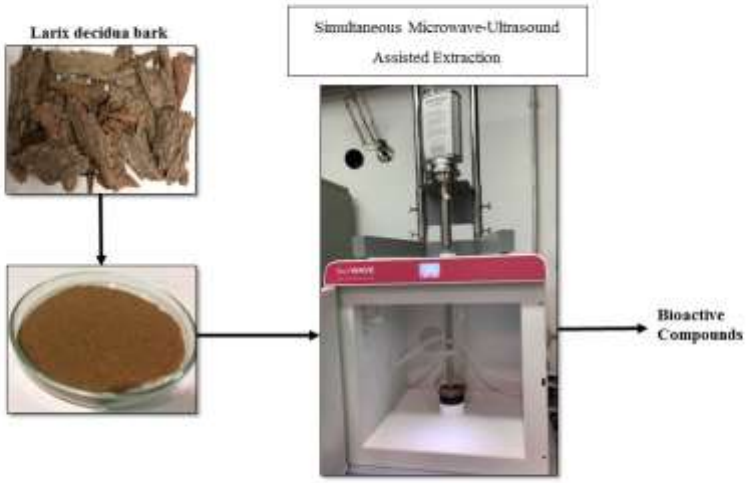
Graphical abstract



Highlights

- Larix decidua bark has high extractive content.
- Extraction yield was optimized, the optimum yield reached 15.72%.
- Optimum point correspond to the highest extraction yield, TPC and TFC.
- SMUAE doubles the extraction yield and reduces the extraction time 47 times.

Abstract

Larix decidua is one of the fastest growing conifers and one of the most important coniferous tree species in Europe with high importance at wood-based industry; however, the bark generated from the debarking process is mainly considered as waste. This study aimed to evaluate the chemical composition and the potential of the extracts of Larix decidua bark as well as the optimization of simultaneous microwave-ultrasound assisted extraction. For this purpose, the Box-Behnken experimental design was used to evaluate the effect of extraction time, microwave power and ultrasound amplitude on the response of extraction yield. The characterization results showed high content of extractives apart from high content in phenolic compounds and high antioxidant capacity, which evidenced the potential of the raw material. Under optimal extraction conditions, 15.72% of extraction yield was achieve. This extract was characterized by total phenolic content, total flavonoid content, antioxidant capacities (DPPH, ABTS and FRAP), high performance size exclusion chromatography and FTIR. Using simultaneous microwave-ultrasound assisted extraction increases the extraction yield to double, having also a better antioxidant capacity, and reduces the extraction time 47 times.

Keywords: bark, simultaneous microwave-ultrasound, extraction, optimization, antioxidant, phenolic compounds

1. Introduction

Trees are mainly composed by wood and bark and they are the main source of wood industry where bark is considered a waste. Hence, bark could be considered as accessible and cheap feedstock [1] since it represents about 9-15% of the total value of the tree [2]. Bark is a heterogeneous material mostly composed by high-molecular polymeric materials (cellulose, lignin and hemicellulose), but also by primary and secondary metabolites [3]. Tree barks are considered as an attractive potential feedstock for biorefinery due to the diverse of its chemical structure which is rich on extractives and polyphenolic compounds [4].

The valorization of barks is a difficult task due to the complexity of its structure. Their compositions depend not only on the tree species but also on environmental conditions [5]. A large amount of organic chemicals can be isolated from bark. Some of these compounds are bioactive, typically produced as secondary metabolites, which are the once that help plants to increase their ability to survive and overcome different challenges [6]. These compounds could be applied in wide range of applications from chemicals and pharmaceutical to green polymers and bio-based materials [7,8]. In recent years, the use of natural antioxidants in the food industry was widely studies, mainly due to the concern for the safety of synthetic antioxidants [9]. The concentration of bioactive compounds present in bark is low; therefore, it is essential to search for selective and efficient extraction process.

Larix decidua (European larch) is one of the fastest growing conifer and it is one of the most important coniferous in Europe [10]. It is very important for wood-based industry due to its properties, such as water-resistant and high durability, good fiber characteristics and low susceptibility to pests [10,11].

One of the biggest challenge of biorefinery is the separation of the biomass compounds. The application of the well stablished “5-Stages Universal Recovery Process” methodology [12] could provide an effective solution for this issue. Extraction is the most important [13] and cost demanding step of separation, corresponding up to 40-80% of the total cost of currently used chemical processes [14]. Conventional extraction methods (Soxhlet extraction and maceration, among others) have some limitations such as: high-solvent cosumption, long extraction time and the possibility of the degradation of target compounds due to the extraction conditions [15]. The extraction technique and the solvent are the two main factors to maximize the selectivity [16]. Many modern non-conventional extraction techniques has been developed which provided a reduction of energy consuption, higher efficiency, higher yield, better temperature control and better quality extracts [17], hence they are considered as sustainable extraction techniques [18].

Microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE) are the most promising techniques. MAE, which uses electromagnetic irradiation, heats by two different mechanisms: dielectric heating, generated by the rotation of the dipole moment, and ionic conduction [16]. MAE has different advantages such as quicker heating, higher extraction yield, lower thermal gradient, smaller equipment size and lower solvent requirements [16,19,20]. UAE, defined as inaudible sound waves at frequency over 20 kHz, is based on the cavitation phenomenon, which involves bubbles formation and collapse. The main advantages of UAE are the reduction of extraction time, solvent and energy consumption, and also the increase of the extraction yield [6]. The acceleration of extraction process with a reduction of extraction time can be performed through the use of simultaneous microwave and ultrasound irradiation due to the synergetic effect induced by the enhancement of mass (ultrasound) and heat

(microwave) transfer [15]. The reduction of the reaction time coupled with the use of green solvents makes this method more environmentally friendly.

In this work, the characterization of Larix Decidua bark and the effect of different extraction parameters were investigated by the optimization of simultaneous microwave-ultrasound assisted extraction (SMUAE) from the bark using a Box-Behnken design. The optimization was carried out in terms of extraction yield and total phenolic content, in order to obtain bioactive compounds using a sustainable techniques. The extracts of the optimal conditions were characterized in term of total phenolic content (TPC), total flavonoid content (TFC), antioxidant activities (DPPH, ABTS and FRAP), molecular weight, and FTIR.

2. Material and methods

2.1 Chemicals

Fisher Scientific supplied dichloromethane, dimethylformamide, tetrahydrofuran and methanol. Gallic acid, Folin-Ciocalteu´s phenol reagent, sodium carbonate and ethanol absolute (synthesis grade) was obtained from Scharlau. Acros Organics supplied iron (III) chloride hexahydrate, barium carbonate and sodium methoxide solution in methanol. Trolox, Aluminum chloride hexahydrate, 2,2-diphenyl-2- picrylhydrazyl hydrate (DPPH), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), 2,2′-azino-bis (3-ethylbenzothiazoline-6 sulphonic acid) (ABTS) and Catechin hydrate were obtained from Sigma-Aldrich. Panreac AppliChem supplied sodium hydroxide, potassium di-hydrogen phosphate, sodium chloride, acetic acid glacial technical grade, potassium chloride, sodium phosphate dibasic, sodium acetate, sulfuric acid, hydrochloric acid 37% and potassium peroxodisulphate.

2.2 Raw material

Larix Decidua bark was supplied by Errekondo Egur-Zerra company (Basque Country, Spain). Before milling, bark was air-dried at room temperature, clay and moss were manually removed and it was cleaned with compressed air. Then a Retsch SM 2000 (Germany) cutting mill was used to ground and sieves to 0.5 x 0.5 mm particle size. It was stored at room temperature in a dark place until their processing.

2.3 Chemical characterization of bark

Chemical composition of raw material was done according to standard methods as well as traditional methods. Moisture, ash content, lignin and carbohydrate content measurements were carried out according to Technical Report of National Renewable Energy Laboratory (NREL), TP-510-42621, TP-510-42622, TP-510-42618 respectively. The suberin content was determined using the method reported by Sillero [21], which is a modification of Pereira´s method [22]. For the extractive measurements sequential soxhlet extraction were carried out with CH2Cl2, EtOH and distilled water for 6, 16 and 16 h respectively following the method described by Miranda [23]. The monosaccharides (mannose, glucose, galactose, xylose and arabinose) determination was done as described Davila [24], and the determination of galacturonic acid, acetic acid and degradation products (furfural and hydroxymethylfurfural) was carried out following the procedure used by Dávila [25].

2.4 Analysis of the bark extracts

The antioxidant capacity and phenolic content were characterized according to the following methodology. First, EtOH/H_2O extraction was performed following one of the most used method. Briefly, EtOH/H_2O (50/50 (v/v)) extraction was carried out with a solid/liquid ratio of 1/10 (w/v) in an ultrasound bath during 1 h at 50 ºC [21,23,26–

28]. The obtained extracts were characterized calculating the yield of the extraction and measuring total phenolic content (TPC), total flavonoids content (TFC), DPPH, ABTS and FRAP. The extraction yield was determined gravimetrically, and its values were presented as means of triplicate analysis referenced to 100 g of dried bark. TPC was determined by Folin–Ciocalteu method [29] and TFC by an Aluminum chloride colorimetric assay [27]. Gallic acid and Catechin were used as standards, the results were expressed as mg of gallic acid equivalents (GAE) /g of dried bark extract, and catechin equivalents (CE) /g of dried bark extract, respectively.

To have an overall understanding of the antioxidant capacity three different methods were used: DPPH, ABTS and FRAP. They are based on the color change made during the reaction of specific radical with the extract measured by UV-vis spectroscopy (Jasco V- 630 UV-VIS spectrophotometer). In all of them Trolox was used as standard and the results were expressed as mg of Trolox equivalent (TE)/g of dried bark extract. Methodology of Gullón [30] was used for the DPPH measurement. FRAP assay was performed according to the Benzie methodology [31]. Finally, the methodology described by Re was used to measure ABTS assay [32].

2.5 Simultaneous microwave-ultrasound assisted extraction

Simultaneous microwave-ultrasound assisted extraction (SMUAE) was performed in an open vessel microwave (MILESTONE flexiWAVE) under reflux with an added ultrasonic unit (HIELSCHER UIP500hdT). The extractions were carried out using Larix decidua bark with a particle size below 0.5 x 0.5 mm, ethanol/water (50/50 (v/v)) mixture as solvent and fixed solid/liquid ratio of 1/10 (w/v). 10 g of dried bark were placed in a 500 mL borosilicate round bottomed flask with 100 mL of solvent and medium stirring level. Before the extraction, the extracts were filtrated through filter

paper under vacuum and then centrifuged. The yield of the extraction was determined gravimetrically and referenced to a 100 g of dried pine bark.

2.6 Experimental design and statistical analysis

The variation on extraction yield and total phenolic content (TPC) were studied changing the values of the microwave power (W), extraction time (seconds) and the ultrasound amplitude (%). The studied variables and their values were selected based on other related research [15,19,33] and preliminary studies (data not show). In all experiments, the maximum temperature reached was always lower than the boiling temperature. The experimental design and the optimization were carried out using response surface methodology with a Box-Behnken design including three replicates in the central point. The variables of this study are reported in Table 1.

Variable	Definition	Unit	Value or range
Fixed	solid/liquid ratio	w/v	1/10
	Solvent: ethanol/water	v/v	50/50
	Shaking speed	%	40
Independent	Extraction time*	seconds	30-120
	Microwave power	W	100-300
	Ultrasound amplitude	%	0-100
Dependent	Extraction yield	%	
	Total phenolic content	mg GAE/g dried bark extract	

*The extraction time is measured as the time that the sample is being exposed to the effects of microwave and ultrasound.

Table 1. Experimental variables used for the optimization.

Statgraphics Centurion XV.II (Statpoint Technologies Inc., Warrenton, VA, USA) software was used to generate the experimental design and the optimization. A second-order polynomial equation was used to fit the data.

$$y_j = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum \sum_{i<j=1}^{k} \beta_{ij} x_{ix_j} + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \varepsilon \quad (1)$$

where y is the predicted response, β_0 is the constant coefficient, β_i, β_{ij}, and β_{ii} are the coefficient of interaction, linear and quadratic respectively, and x_i and x_j are the independent variables. The suitability of the model was determined by the evaluation of the coefficient of determination (R^2), the significance of the regression coefficients, and the F-test value obtained from the analysis of variance.

With the aim of optimizing the selected response variables simultaneously, a multiple response surface optimization was conducted. The selection criteria were relied on obtaining the highest extraction yield in addition to a high TPC in the defined range of conditions. A comparison between the experimental values obtained at the optimal point and the ones predicted by the model was done for the validation of the model.

2.7 Characterization of bark extracts in optimal conditions

2.7.1 Determination of phenolic content and antioxidant activities

Total phenolic content, total flavonoid content and the antioxidant activities (DPPH, ABTS and FRAP) were determined by methods described in detail in the section 2.4 of this documents.

2.7.2 Determination of molecular weight distribution

High performance size exclusion chromatography (HPSEC) was used to analyze the Mw, Mn and Mw/Mn of the isolated extractives following the method described by Dávila [25].

2.7.3 Infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) was used to analyze the main chemical functionalities of the extracts. It was determined on a PerkingElmer Spectrum Two spectrometer fitted with a Universal Attenuated Total Reflectance accessory. The defined working range was from 700 to 4000 cm^{-1} with 4 cm^{-1} resolution with 12 registered scans.

3. Results and discussion

3.1 Larix decidua composition

As far as we know, no one had previously implemented the characterization of Larix decidua bark. Thus, the raw material was preliminary characterized in order to determine the composition and the potential uses.

3.1.1 Chemical composition of Larix decidua bark

The chemical composition of Larix decidua bark was carried out obtaining the values reported in the Table 2. Total lignin (composed by klason and acid soluble lignin) is the main component of the pine bark (36.8%). Similar percentages were reported by Nunes, and Ruiz-Aquino for Pinus pinea, (37.5%) and Quercus laurina outer bark (36.9%) [34,35]. Cellulose content, measured as glucan content, was the second main component followed by the total extractive content that resulted in a 20.44% of the dry weight of the raw material. This content is analogous to the values obtained by Miranda for spruce bark (20.6%) and Ruiz-Aquino for Quercus laurina outer bark (19.3%) [35,36]. The

total content in polysaccharides, understanding this as the sum of the content in cellulose and hemicellulose, is 33.3%, which is a similar value that the one reported by Sillero for Sweet chestnut (34.6%) [21]. The value for suberin content was similar to the result reported by Nunes for Pinus pinea (2.5%) [34]. In general, the total amount of non-extractable compounds (lignin, suberin, cellulose and inorganic compounds) is 68.1% of the total of the barks. Although in the bark the extractive part is higher than in other parts of the tree, it continues to exist in less quantity than the non-extractable part. In this way, it is reflected the need to choose the most suitable extraction treatment.

Component	% original dry mass
Ash	3.5±0.1
Extractives total	20±1
Dichloromethane	1.9±0.1
Ethanol	8.7±0.7
Water	9.9±0.7
Suberin	2.0±0.2
Lignin total	36.8±0.1
Klason lignin	33.1±0.2
Acid soluble lignin	3.71±0.04
Cellulose[a]	25.7±0.6
Hemicellulose[b]	7.6±0.9

[a] Represented as glucan content
[b] Measured as the join contribution of the rest of the sugars

Table 2. Summative chemical composition of Larix decidua bark.

3.1.2 Characterization of bark extract

With the aim of understanding the real potential for obtaining biologically active compounds from the pine bark, the quantification of phenolic and polyphenolic compounds as well as antioxidant capacity of bark extract were carried out as can be

seen in Table 3. The fact that they show antioxidant activity makes them suitable for their use against oxidation and degradation in a variety of applications in different industries such as pharmaceutical, and food preservation among others.

	Extract characterization	SMUAE (optimal conditions)	Conventional extraction
Extraction yield (%)	20±1	15.1±0.1	7.7±0.4
Total phenolic content (mg GAE/g dried bark extract)	538±7	596±19	567±24
Total flavonoid content (mg CE/g dried bark extract)	593±46	433±17	417±16
Antioxidant capacities			
DPPH (mg TE/g dried bark extract)	636±33	838±6	749±26
ABTS (mg TE/g dried bark extract)	1040±41	1178±21	807±7
FRAP (mg TE/g dried bark extract)	441±5	459±17	330±30

Table 3. Characterization of different bark extracts; extract for the characterization of the raw material, extracts obtained under optimal conditions and extracts obtained by conventional extraction [33].

The extraction yield was similar to the total extractive content determined by sequential extraction for the characterization (Table 2). Thus, the suitability of the extraction method for the characterization of the extracts as well as the high content of extracts of the bark is confirmed.

The phenolic and polyphenolic nature of the extracts is demonstrated due to the high content in phenols and flavonoids. The value obtained for TFC, 593 mg CE/g dried bark extract, is greater than other values obtained for the extracts of other raw materials. It is about a quarter higher than the one obtained for ethanolic extracts of Pidea abies bark by Neiva, 476 mg CE/g extract [37]. The difference is higher for ethanol-water extracts

of other raw materials such as Goupia glabra and Quercus fanginea, (74.8 and 204.72 mg CE/g extract, respectively) [26,28]. The value obtained for TPC is similar to the values reported by Chen and Carmo for Acacia mearnsii and Copaifera langsdorffii, respectively [38,39].

Focusing on the antioxidant capacities measured for the extracts it can be concluded that the three measurement provide good antioxidant capacities. DPPH value is similar to the one reported by Miranda for E.sideroxylon (648 mg TE/g dried bark extract) and lower than the values obtained for Q. fanginea and Albizia niopoides [23,26,40]. There are few results where ABTS and FRAP antioxidant capacities are described for bark extracts. But if we compare the value measured for both parameters with those reported by Sillero for six different bark extracts, it can be concluded that these are within the ranges defined in that article [21].

The characterisation results evidence that bark is a promising feedstock. Therefore, the possibility to obtain bioactive compounds is confirmed.

3.2 Simultaneous microwave-ultrasound assisted extraction (SMUAE) conditions of Larix decidua bark

Based on the chemical composition and the characterization of the extracts made in the previous section, we consider that this raw material is suitable for bioactive compounds extraction. In order to explore this possibility, different experiments have been carried out to determine the optimum point at which extraction yield and total phenolic content (TPC) are maximized.

No bibliographic evidence has been found for the combination of the two methods for bark extractions. MAE and UAE are relatively new extraction technologies, and the use of both techniques simultaneously is not yet very well developed, due, perhaps, to

technical difficulties. However, the synergy generated by the use of both techniques simultaneously could be beneficial for extractions. In following section, the results obtained for the experimental design of SMUAE of pine bark are analyzed and discussed.

The discussion of the results of the optimization of TPC was not considered due to the low variability of the values measured throughout the experiments. The values varied between 572 and 617 mg GAE/g dried bark extract, which correspond to less than 8% of variability. In addition, the obtained results are in the range of the one measured in the characterization. Therefore, it can be concluded that the parameters studied in this work does not have a direct effect on TPC of the extracts.

3.2.1 Modelling and optimization of SMUAE conditions

Table 4 presents the 15 experiments performed for the Box-Behnken experimental design along with the obtained experimental results. The fit of the model was evaluated by analysis of variance (ANOVA). Table S1 in the Supplementary data shows the regression coefficient and the statistical parameters obtained for the model. The measured statistical are determination coefficient (R^2), Student's t-test for statistical significance and Fisher's F test for the models' statistical significance. The determination coefficient value (0.854) obtained for the evaluated response indicate the competency of the model. Fig. 1 presents predicted versus experiment graphs for extraction yield. The point grouped around the diagonal line indicates a proper fit of the model.

Nº Exp	X_1	X_2	X_3	Yield (%)
1	75	200	50	12.94
2	30	200	0	12.25

3	75	100	100	14.57
4	120	200	100	15.64
5	30	300	50	13.42
6	75	100	0	11.68
7	120	200	0	13.13
8	75	200	50	13.07
9	120	100	50	13.26
10	30	200	100	10.66
11	75	200	50	14.11
12	120	300	50	14.44
13	30	100	50	11.76
14	75	300	-0	13.62
15	75	300	100	15.22

Table 4. Tested operational conditions expressed in terms of dimensionless and dimensional independent variables (X_1 (extraction time, sec), X_2 (microwave power, W) and X_3 (Ultrasound amplitude, %)) and their response.

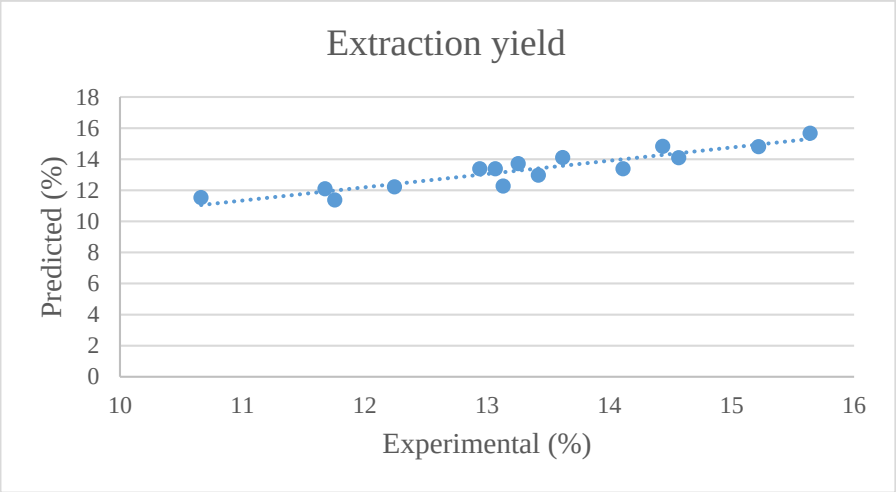


Fig. 1: Comparison of predicted and experimental data of extraction yield.

Second-order polynomial equation has been calculated for the extraction yield using the multiple regression analysis of the experimental data. It is expressed as follow:

$$Yield=13.37+1.05X_1+0.68X_2+0.68X_3-0.50X_1^2-0.12X_1X_2+1.03X_1X_3+0.35X_2^2-0.32X_2X_3+0.05X_3^2 \quad (2)$$

3.2.2 Effect of the independent variables on extraction yield.

The results show that the extraction yield varied between 10.66% and 15.64%, experiments 10 and 4 respectively (Table 4). That indicates that the treatment conditions greatly influenced the extraction yield, with a variability higher than 30%.

According to the regression coefficients (Table S1, Supplementary data), the linear effect of the three independent variables, just as the interaction effect between extraction yield and ultrasound amplitude were the variables that exercised a significant influence on extraction yield. The interaction between extraction time and microwave power, and also microwave power and ultrasound amplitude did not have a significant effect on the response, as it can be seen in Table S1 (see Supplementary data) and Eq. (2). Our results cannot be properly compared with other studies because as far as we know, there are no SMUAE for tree bark extractions. But if we compare them with the results obtained by Jha for black rice husk at a sequential ultrasound-microwave extraction, we can say that our results are in agreement with theirs results in regards to the importance of the extraction time [41]. Comparing the results with the SMUAE studied by Luo for walnut flour, there is a coincidence in the importance that microwave power and extraction time have, and also in the significance of the interaction of ultrasound and extraction time [15].

The interaction effects of microwave power and ultrasound amplitude in the extraction yield for a fixed middle point value of extraction time 75 sec (X_1= 0) is showed in Fig. 2a. In this plot, it can be noticed that the maximum extraction yield value was achieved for the maximum microwave power and ultrasound amplitude. Nevertheless, for the maximum value of ultrasound amplitude and with 100 W of microwave power the

obtained extraction yield was high. In addition, it was observed that for low microwave power the effect of the ultrasound amplitude is large, while when the maximum power of microwave was used the effect of the ultrasounds is reduced. In the graph, you can also see the influence of the microwave power looking at the part where the ultrasound amplitude is set in 0%. It is seen that the increase in the extraction yield has an almost linear growth of up to 2% of extraction yield, but without reaching the maximum value.

Fig. 2b allows to visualize the interaction between extraction time and ultrasound amplitude keeping the microwave power constant at 200 W (X_2=0). This relation has the highest significance level, so is the relation with the greatest influence on the optimization. As it can be seen, with the shortest extraction time the influence of the ultrasound is low. This could be due to the lack of time for cell disruption that should be generated as a result of the application of ultrasound. Moreover, when the amplitude of the ultrasound is the maximum, the extraction yield increases almost linearly reaching its maximum value at maximum extraction time and ultrasound amplitude. This increase is close to 4% of extraction yield and is generated by increasing the extraction time with the maximum ultrasound amplitude. It confirms the hypothesis that it takes a minimum time to have an efficient cell disruption.

In Fig. 2c can be seen the response surface in function of extraction time and microwave power for a constant value of ultrasound amplitude (X_3=0). It can be noted, that for the minimum microwave power (100 W) and maximum extraction time (120 sec) the obtained extraction yield is high, and it is improved raising the microwave power. It can be also seen how there is a large increase, greater than 1% of extraction yield, when the minimum time was used and the microwave power is increased from 100 to 300 W. Finally, on the plot you can see how, unlike ultrasound amplitude, the microwave power does affect the extraction yield at low times.

Taking into account all the analyzed results, it can be concluded that the bark extraction yield is enhanced by the use of simultaneous microwave-ultrasound assisted extraction. That could be due to the synergy generated by the use of microwave and ultrasound techniques at the same time.

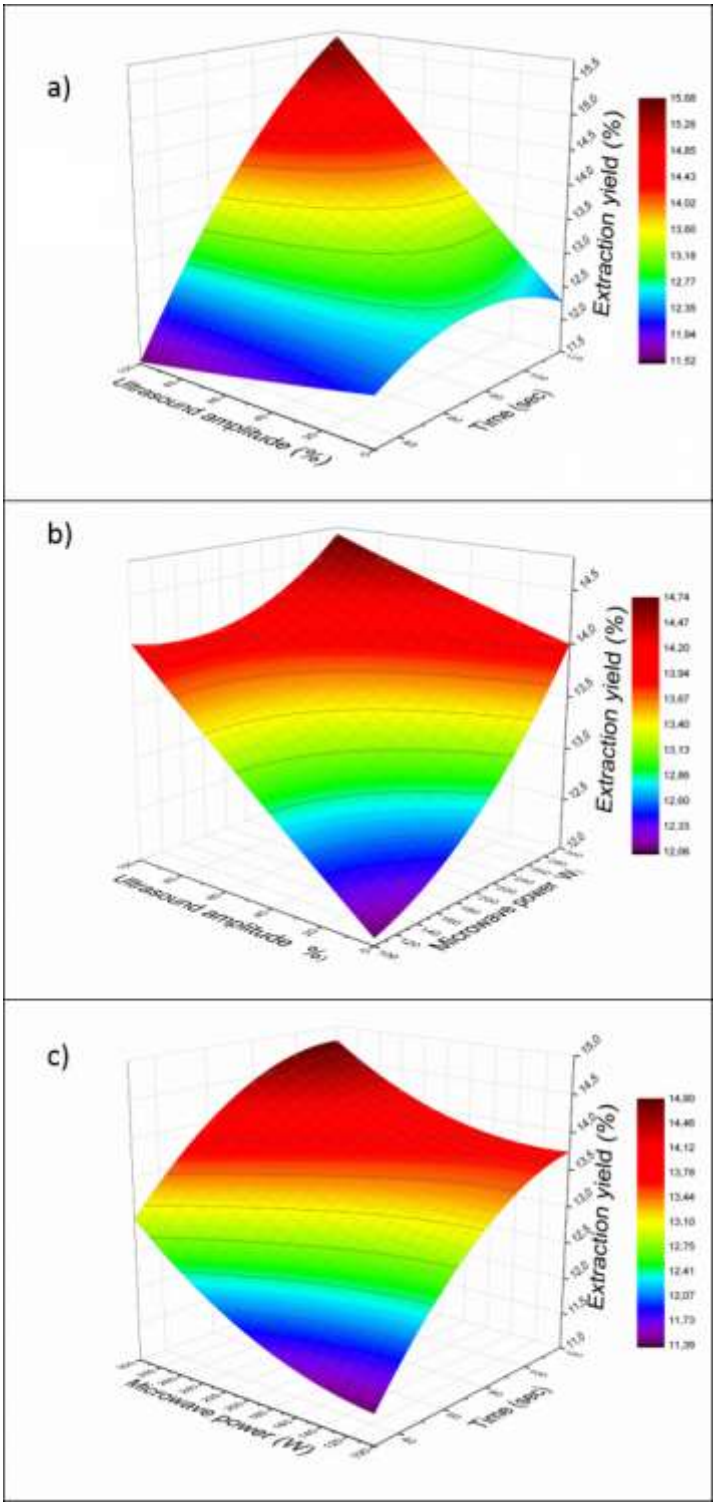


Fig. 2: Response surface plots for extraction yield. (a) Microwave power and Ultrasound amplitude at a fixed extraction time (X_1=0); (b) Extraction time and Ultrasound amplitude at a fixed microwave power (X_2=0); (c) Microwave power and Ultrasound amplitude at a fixed ultrasound amplitude (X_3=0).

3.3 Optimization of extraction conditions and validation of the model

The optimization of SMUAE to achieve the maximum extraction yield that would simultaneously provide the greatest total phenolic content (TPC) was carried out using Statgraphics Centurion XV.II software. TPC was not considered in that optimization due to the reasons explained above. The model predicted the maximum extraction yield (16.25%), which correspond nearly to the highest extraction time (119.95 sec), microwave power (300 W) and ultrasound amplitude (99.68%).

To validate the model, three experiments were performed under the optimum conditions. The adequacy of the model for quantitative predictions was validated by the successful agreement between the measure and the predicted value. The experimental mean value of extraction yield was 15.72 ± 0.08%, which was close to the predicted value of 16.25% from the model. This fact confirms the suitability of the response surface methodology.

3.4 Characterization of bark extract in optimal conditions

3.4.1 Extraction

Looking at the extraction yield obtained for the optimum point it can be concluded that the obtained yield was close to the total extractive content determined by sequential extraction for the characterization of the raw material (Table 3). Thus, the suitability of the extraction method as well as the high content of extracts of the bark is confirmed. Comparing the value obtained for SMUAE with that obtained using conventional extraction (CE) method (see Table 3); an improvement in the extraction yield of the

double is observed. This increase is very good, extracting around 75% of the total extractive content of the raw material. The obtained extraction yield is greater than the values reported by Sillero [33] for separate extractions with ultrasound (5.87%) and microwave (8.21%). It also improves the values of other studies carried out with microwave-assisted extraction (MAE) for maritime pine bark and spruce bark [42,43]. Therefore, it can be concluded that the use of both techniques simultaneously improves the extraction yield. This is due to the synergetic effect induced by the simultaneous use of microwave and ultrasound irradiation [15]. As a result of this effect, the reaction time is considerably reduced by the use of a single operation unit, thus complying with one of the principles of green extraction [44].

3.4.2 Total phenolic content (TPC) and Total flavonoid content (TFC)

The total phenolic and flavonoid content for the SMUAE are presented in Table 3. The TPC value is higher than that obtained for the characterization of pine bark extracts as well as for the CE (see Table 3). Nor are large variations observed in the values obtained through MAE and UAE for the same raw material, 525 ± 3 and 579 ± 21 mg GAE/g dried bark extract respectively [33]. The obtained value for SMUAE is higher than that reported for maritime pine bark by Chupin [42]. The maximum value obtained in this work using MAE is 306 ± 33 mg GAE/g extract, far below the value that have been obtained in this work by combining microwave and ultrasound.

As in the case of the TPC, there is no improvement for the TFC with respect to conventional extraction, as seen in Table 3. Furthermore, the value obtained is below the total potential of the bark, which was measured in the characterization of the bark extracts. However, this is better than those reported in other works. Comparing these results with those obtained by Chupin for the bark of maritime pine [42], it can be

concluded that a better TFC is obtained, being the value obtained of MAE of the maritime pine bark 403 ± 42mg GAE/ dry plant.

Taking into account all the above, it can be concluded that the TPC and the TFC seem not to be greatly influenced by the extraction method used for this pine bark. This confirms that the SMUAE, at the optimized conditions, does not generate degradation of the extracts.

3.4.3 Antioxidant capacity

Table 3 shows a summary of the results obtained for the analysis of the antioxidant capacities of the extracts. The values obtained with SMUAE for the three antioxidant capacities under study (DPPH, ABTS and FRAP) are higher than those obtained by conventional extraction (CE) method as well as for the characterization of the extracts of raw material. The obtained scavenging capacity against the radical DPPH was close to 100 mg TE/g dried bark extract higher than the value obtained for CE. In the case of ABTS, the results obtained were better, since the value obtained for SMUAE is increased respect to CE by more than a third of the total value obtained for CE. Finally, in the case of FRAP, the increase in the value obtained for SMUAE is of the same magnitude as that given for ABTS.

There are few results in the literature regarding the antioxidant capacity of these types of extractions performed to tree bark, and in general, the only one used is DPPH. Due to that, the comparison of the results with other literature data is not easy, and it should be done carefully. Comparing the results previously obtained by Sillero [33] for DPPH by MAE and UAE for the same raw material used in this study with the one measured by SMUAE, higher value is observed. Both results obtained previously for MAE and UAE have quite similar results to each other (748 ± 38and 750 ± 37 mg TE/g dried bark

extract respectively), while the value obtained in this study exceeds it by more than 100 mg TE/g dried bark extract. If we compare the results with those of another raw material, in this case Morus nigra leaves, it can be seen that the one obtained by SMUAE is considerably higher. Radojković reported a range of values for DPPH between 11 and 18 mg TE/ g dry plant [45], while the value obtained for SMUAE (123 ± 2 mg TE/g dry bark) can be up to 10 times greater.

The values reported by Sillero [33] for ABTS and FRAP of the extracts obtained through MAE and UAE are also lower than the values calculated in this study. In the case of ABTS, the results reported for MAE was 906 ± 31 mg TE/g dry bark extract, and that reported for UAE was 677 ± 35 mg TE/g dry bark extract. These results are considerably lower than those obtained in this work. Zoumpoulakis reported 22.83 mg TE/g dry extract for the ABTS antioxidant capacity of the commercial antioxidant BHT, which is 18 times lower than that obtained for the SMUAE extracts [46]. The values reported by Sillero for FRAP (390 ± 9 mg TE/g dry bark extract for MAE and 351 ± 29 mg TE/g dry bark extract for UAE) are not so different from that obtained by SMUAE, although they remain lower [33].

The evidence of antioxidant activity makes the obtained products suitable for use against oxidation and degradation in a variety of applications. It can be concluded that using SMUAE the antioxidant capacity of the extracts is improved, showing the potential use of Larix decidua bark as a promising antioxidant source in different industries such as agri-food, pharmaceutical and cosmetic among others. The use of antioxidant compounds in sunscreens improves their properties [47], and they can also be used in the food industry to protect against food degradation [9, 48]. However, the use of the compounds obtained in this work in food must be studied in more detail prior

to their use, particularly their toxic effects, interaction with the food and their effect on organoleptic properties of food.

3.4.4 Determination of the molecular weight distribution

Fig. S1 (see Supplementary data) illustrates the molecular weight distribution of the extract obtained from Larix decidua bark under the optimal condition. As can be seen in that figure, the extract consisted of a heterogeneous mixture of compounds divided into different weight fractions. The difference in weights may be due to a difference in the degree of polymerization of the compounds in the extract [49].

					Global average		
		Mw	Mn		Mw	Mn	
	Percentage	(g/mol)	(g/mol)	Mw/Mn	(g/mol)	(g/mol)	Mw/Mn
	82.21	20446	8221	2.49	16939	2287	7.41
	10.26	1016	933	1.09			
Larix	5.97	392	370	1.06			
decidua	1.57	237	237	1.00			

Table 5. Percentage, average molecular weight (Mw), number average (Mn) and polydispersity index (Mw/Mn) of EtOH/H_2O bark extract under optimal conditions.

As it can be seen in Table 5, the global polydispersity index of the extract is far from the value 1. This is because there are considerable differences in the molecular weights of the compounds present in the extracts. Due to the extractions are carried out at low temperature, in the extract it can be found from monomers and dimers of low molecular weight, to oligomers and high molecular weight flavonoids. More than the 82% of the compounds have an Mw of 20.45 g/mol, what it means that the degree of polymerization is high. However, the rest of the compounds have a much smaller Mw,

below 1000 g/mol. This distribution is in the same range as the ones reported by Sillero for the characterization of extracts from different tree barks [21]. In that article, extracts of up to 6 different barks are characterized, of which 4 obtain a global Mw similar or higher than that obtained in this study. The Sweet chestnut has the highest global Mw (57.39 g/mol), while the Northern red oak, the common oak and the Iberian withe birch have it lower (17.21 g/mol; 20.23 g/mol and 30.97 g/mol, respectively). In the case of the Sweet chestnut and the Iberian white birch, they also have less than 20% of the compounds with Mw below 1000 g/mol.

3.4.5 Structural characterization of the extract by FTIR

The spectra of the extract are presented in the Fig. S2 (see Supplementary data). The band assignation is relying on the assignments given by Boeriu, Ping, Soto and Chupin [42,50–52].

According to the band assignment, the bands with wavelengths smaller than 900 cm^{-1} are assigned to –CH stretch vibration. Aromatic –CH bending in-plane vibration is detected at 1105 cm^{-1}. The bands 1200 cm^{-1} and 1050 cm^{-1} can be attributed to C-O stretching vibration. The peak 1275 cm^{-1} is assigned to a C-O-C asymmetric stretch vibration [53]. The bands at 1605 cm^{-1}, 1515 cm^{-1} and 1440 cm^{-1} are originated from aromatic skeleton vibration. 1440 cm^{-1} also correspond to –CH deformation. The peak at 2925 cm^{-1} is identified as –CH stretch vibration in aromatic methoxy groups and in methyl and methylene groups of side chains. Finally, the band at 3300 cm^{-1} is attributed to –OH stretch vibration in phenolic and aliphatic structure.

The band corresponding to –OH in phenolic and aliphatic structure is very big, which confirmed the high activity of the sample measured by antioxidant capacities. The peaks

assigned to different aromatic structure vibration ratified the high content on phenolic compounds of the extract.

4. Conclusions

The characterization of Larix Decidua bark as well as the optimization of the extraction conditions for the extraction of bioactive compounds from the bark was successfully carried out. The results of the characterization showed that this pine bark is rich in extractives. In addition, the bark can be considered as a source of biomolecules with a high antioxidant capacity. These good results allow considering it as an interesting renewable source with a high potential for valorization.

On the other hand, the results of the optimization carried out with Box-Behnken proved that the interaction between the extraction time and ultrasound amplitude had the greater impact on the extraction yield. Although it has not been possible to optimize the total phenolic content due to its low variability, the optimization of the extraction yield has been carried out correctly. The value predict by the model was consequent with the experimental value, and it is considerably larger than the one obtained by conventional method.

The characterization of the extract obtained under the optimal conditions showed that the extract has a high content not only in phenolic compounds, but also in flavonoids. The content of high molecular weight compounds is also confirmed by HPSEC, since it is observed that the average molecular weight is large. The antioxidant capacities were improved significantly compared to the conventional extraction method. In conclusion, these extracts are more biologically active, which is very good for different applications in fields as varied as food industry, cosmetic or bio-based materials.

Taking all the above into account, it can be concluded that SMUAE is a very good extraction method not only for the extracted good quality extracts, but also for the reduction of extraction time, which is reduced by 47 times. The results obtained in this research confirm the improvement of the competitiveness of the wood industry due to the possibility of using the bark as a natural source of bioactive compounds and the generation of economic value to the waste through the use of sustainable innovative extraction technique.

CRediT author statement

Leyre Selliero.: Investigation, Data curation, Formal analysis, Writing- Original draft preparation. **Raquel Prado**: Methodology, Supervision, Writing- Reviewing and Editing. **Jalel Labidi**: Conceptualization, Funding acquisition, Writing- Reviewing and Editing,

Declaration of interest

The authors declare no conflict of interest.

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