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Optimization of Different Extraction Methods to Obtaining Bioactive Compounds from Larix Decidua Bark

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Bark is considered as waste generated by wood processing industries. Bark is being used for horticulture applications and energy production, but due to its complex structure, bark can become biorenewable source. It is rich in high add-value compounds, as bioactive compounds, which are used in medicine, nutrition or personal care products. The most used extraction method for this bio-compounds is the conventional (liquid extraction), but in recent years others are being investigated. In this work conventional extraction (CE), ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE) were optimized to obtain the highest extraction yield (%). In the optimal point of the design, total phenolic content (TPC) and total flavonoids content (TFC) of the extract of each method were determined. Their antioxidant capacity was also measured using 2,2-diphenyl-1-picrylhydrazyl assay (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay (ABTS) and ferric reducing antioxidant power (FRAP) assay. The best extraction yield (%) as well as the highest antioxidant capacities were obtained with microwaves. The results showed the potential of the bark of Larix Decidua as source of bioactive compounds. Using microwaves for extraction could save energy and reduce the processing time.

1. Introduction

The interest in the use of natural products obtained from renewable sources instead of the synthetics ones obtained from fossil fuels is increasing because of the concern of the society about the preservation of the environment and their impact in the health care. Thus, the biorefinery approach is extending, what implies that bio-renewable sources are used as an alternative to obtain the new environmentally friendly compounds for wide applications such as cosmetic, medicines and personal care products.

Around 1,000 ha of forest are dedicated to produce a range of materials every year (FAO, 2016). Considering bark as a waste and that it comprised about 9-15 % of the total volume of the tree (Leite and Pereira, 2017), it can be considered as a high waste problem. Nowadays the main uses of the bark are for horticulture applications and energy production. Caloric value is higher for bark than for actual wood but still is not enough to compete with the fossil fuel. Moreover, bark combustion can damage the combustors because of its high content of ash and the low sintering point of bark ash (Feng et al., 2013). All these facts affect to economy feasibility of bark combustion. Because of that, in the last years different alternatives are being explored in order to assess the potential uses of these by-products in biorefinery.

Bark is chemically composed by lignin, cellulose, suberin, extractives (waxes, fatty acids, terpenes, flavonoids, lignans, tannins and extractable carbohydrates) and inorganic compounds (Dou et al., 2016). The complexity of bark structure which had made difficult its decomposition, bark can be a source of a huge amount of compounds with high add value (Harkin and Rowe, 1971) as bioactive compounds, which are attractive for fields of nutrition, medicine and health. The possibility of isolate this compound has caught the eye of researcher around the world, and the number of reports from groups working in the field has increase.

Bioactive compounds are mainly produced by secondary plants metabolites. Some of them have antioxidant capacity, which is very important for plant defence mechanism. Amorati and Valgimigli define an antioxidant as a substance that, when it is added to an oxidable molecule in small amount is able to protect such molecules

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by delaying, retarding or inhibiting their autoxidation (Amorati and Valgimigli, 2015). That ability is desirable to protect molecules against the oxygen reactivity, so they are interesting compounds for pharmaceuticals, nutritionals or cosmetics uses.

The most extended way of extracting bio-compounds is by using conventional extraction, which consist on a solid-liquid extraction using huge quantity of solvents and a long time for the extraction. This technique could be carried out by maceration, applying heat and/or agitation, or with a Soxhlet extractor. Nevertheless, this technique is not an environmentally friendly technique due to the huge amount of solvent and energy consumption. In order to increase the sustainability of the process many different eco-friendly techniques are being investigated. Two of the most investigated techniques are microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE). With both of this technique, the main objectives are the reduction of extraction time, solvent consumption, energy consumption, waste generation, and the increase of the extraction yield (Idris and Sulaiman, 2017). There are many papers related to that topic (Azmir et al., 2013), for example, Hofmann concluded that MAE and UAE can be used for the extraction of antioxidants compounds (Hofmann et al., 2015), and Vázquez find that they are attractive alternatives for the conventional extraction techniques (Vázquez et al., 2014).

In this work, the optimization and comparison of different extraction methods from pine bark (Larix Decidua) were carried out in term of the extraction yield. The selected three techniques for this work were conventional extraction (CE), ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE). Once the optimization was done, the extracts of the optimal conditions of each extraction method were analysed measuring total phenol content (TPC), total flavonoid content (TFC), and antioxidant activities (DPH, ABTS and FRAP).

2. Materials and methods

2.1 Raw material

Larix Decidua bark was recollected at Errekondo Egur-Zerra Company (Basque County, Spain). After the collection, it was air-dried at room temperature until constant moisture. Before milling, the bark was cleaned by pressure air and manually to removed clay and moss. Then it was milled with a cutting mill (Retsch SM 2000, Germany) with 0.5 x 0.5 mm mesh. Finally, it was stored in a dark place at room temperature until it was used.

2.2 Conventional Extraction (CE)

Larix Decidua bark was subjected to an extraction in an orbital shaker (Heidolph Unimax 1010) with temperature control (Heidolph Incubator 1000) using a mixture of ethanol and water (50/50 (v/v)) as solvent. Three grams of dried bark were placed in 100 mL Erlenmeyer flasks using a fixed solid/liquid ratio of 1:8 (w/v) and a shaking speed of 120 rpm. The extracts were filtrated through filter paper under vacuum and the yield of the extraction was calculated gravimetrically and referenced to a 100 g of dried pine bark.

2.3 Ultrasound Assisted Extraction (UAE)

The extraction was carried out in an ultrasound bath with temperature control (Elmasonic 570 H, Elma) using ethanol/water (50/50 (v/v)) mixture as solvent. Three grams of dried bark were placed in a 100 mL Pyrex™ Borosilicate Glass with a fixed solid/liquid ratio of 1:8 (w/v). The extracts were filtrated through filter paper under vacuum and the yield of the extraction was calculated gravimetrically and referenced to a 100 g of dried pine bark.

2.4 Microwave Assisted Extraction (MAE)

MAE was performed in an open vessel microwave (CEM Discover) under reflux using ethanol/water (50/50 (v/v)) mixture as solvent. Three grams of dried bark were placed in a 100 mL round bottomed flasks with a fixed solid/liquid ratio of 1:8 (w/v). Before the extraction, the extracts were filtrated through filter paper under vacuum and the yield of the extraction was calculated gravimetrically and referenced to a 100 g of dried pine bark.

2.5 Experimental design

The effect of different variables on the extraction yield (%) were studied depending on the type of extraction methods. The independent variables are reported in Table 1, where it can be seen that for the CE and UAE the studied variables were time and temperature, while for MAE the studied variables were time and power.

Table 1: Independent variables for the extraction methods and its maximum and minimum values.

Method	Temperature (ºC)		Time (min)		Power (W)	
	Min	Max	Min	Max	Min	Max
CE	40	65	30	180		
UAE	40	65	10	120		
MAE			10	120	100	300

The influence of different operational conditions in the different extraction methods were analyzed using a two-level-two factor experimental design with 10 experiments and 3 replicates of the central point. For the optimization a response surface methodology (RSM) were used to maximize the selected response variable, extraction yield (%). The experimental design and optimization were carried out by the Statgraphics Centurion XV.II software, where the data were fitted using a secondary-order polynomial described by the Eq(1).

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

The suitability of the model was determined by the coefficient of determination (R^2). For the adequacy of statistical significance of the regression coefficients an analysis of variance (ANOVA) was used with a confidence level of 95.0 %. Model validation was implemented by carrying out the experiments at the optimal extraction conditions and making a comparison between the values predicted by the models and the experimental data.

2.6 Determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Folin-Ciocalteu method (Singleton and Rossi Jr., 1965) was used for the determination of the total phenolic content (TPC). Gallic acid was used as referent standard, and the results were expressed as mg of gallic acid equivalents (GAE)/g of dried bark extract. Total flavonoid content (TFC) was determined by an Aluminum chloride colorimetric assay (Lima et al., 2017). Catechin was used as referent standard, and the results were expressed as mg of catechin equivalents (CE)/g of dried bark extract.

2.7 Determination of antioxidant capacity

Three different types of antioxidant capacity assays were measured in order to have a global vision of the real antioxidant capacity of each extract. For all the assays Trolox was used as referent standard and the results were expressed as mg of Trolox equivalent (TE)/g of dried bark extract.

DPPH radical scavenging assay was carried out according to the methodology described by Gullón et al. (2017). Briefly, 3 mL of $6 \cdot 10^{-5}$ M methanolic solution of DPPH was added to 300 µL of a methanolic solution of each sample. The decrease in absorbance at 515 nm after 15 min was measured in a spectrophotometer (Jasco V-630 UV-VIS spectrophotometer).

The ferric reducing antioxidant power (FRAP) assay was performed according to the Benzie described methodology (Benzie and Strain, 1996). Shortly, the reactive solution was prepared with the relation 1:1:10 with 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$ in distilled water and 300 mM acetate buffer (pH 3.6). 3 mL of this reactive solution were mixed with 0.1 mL of a methanolic solution of each sample and after 6 min, the absorbance was measured in a spectrophotometer (Jasco V-630 UV-VIS spectrophotometer) at 593 nm.

ABTS assay was used to measure the Trolox equivalent antioxidant capacity (TEAC) by the methodology of Re (Re et al., 1999). For this method, phosphate buffer saline (PBS) (pH 7.4) and 7 mM ABTS stock solution with 2.45 mM potassium persulfate mixture is used, which have an absorbance of 0.7 at 734 nm. Once the solution is prepared, 3 mL of this ABTS solution was added to 30 µL of each sample and after 6 min, the absorbance was measured in a spectrophotometer (Jasco V-630 UV-VIS spectrophotometer) at 734 nm.

3. Results and discussion

3.1 Yield of the different methods of extraction

In this study conventional extraction (CE), ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE) were used for the extraction of pine (Larix Decidua) bark extracts. Table 2 summarized the experiments design for the three different extractions and the obtained extraction yield. For CE and UAE X_1 correspond to temperature (ºC) values and X_2 correspond to time (minutes), while for MAE X_1 correspond to temperature (ºC), also, but X_2 correspond to microwave power (W).

Table 2: Design and results of response surface method analysis.

Method	CE			UAE			MAE		
	X_1	X_2	% extraction yield	X_1	X_2	% extraction yield	X_1	X_2	% extraction yield
1	1	1	7.78	0	0	6.48	0	0	7.83
2	0	-1	8.23	0	-1	5.71	0	0	7.91
3	0	0	8.01	-1	0	4.57	-1	1	7.81
4	1	0	8.12	-1	1	3.47	1	-1	7,87
5	0	1	7.83	1	0	5.18	-1	-1	8,93
6	-1	1	6.15	1	1	7.33	1	0	7.27
7	0	0	8.71	1	-1	6.04	1	1	4.95
8	-1	0	6.70	0	1	3.71	0	-1	8.96
9	-1	-1	5.50	0	0	7.12	-1	0	6.57
10	1	-1	8.11	-1	-1	6.16	0	1	7.97
Optimal	58.26	94.27	7.73	65	94.76	5.87	62.66	100	8.21

The extraction yields obtained in the different extraction methods techniques were completely different as it can be seen in the Table 2. MAE has the best extraction yield with an 8.21 %, and UAE has the worst extraction yield. These results are in accordance with the date reported in other work (Aspé and Fernández, 2011) which demonstrated that MAE has better extraction yield than Bach extraction and UAE, and that extraction yield is improved when sequential extraction are carried out.

For the conventional extraction (CE) the obtained values were for medium temperature and not too long times. It could be because with too much time the extracted compounds could be degraded, and the same with high temperatures. In the case of ultrasound (UAE), the best results were obtained with the highest temperature and long time. But still with the fixed variables it seems that the system need something more to increase the extraction yield to obtain at least the same as CE, so a higher sonication frequency could be needed. Nevertheless, for microwave (MAE) the lower power and longer time were needed to obtain the best results. It could be because with a higher power could break down the extracted compound (Fernández-Agulló et al., 2015) reducing the amount of extracted compounds and their antioxidant capacity.

3.2 Optimization of the extraction conditions

In order to obtain the best extraction yield (%) an optimization was done with a two-level-two factors method combined with response surface method (RSM). Table 2 summarized the experimental plant according to the RSM. The value determined by R^2 was used to measure de correlation and significance of the models. Taking in to account that the confidence level used was 95.0 %, only the model for CE was significant (95 %), for the other two the R^2 value were less than the required, 60 % for UAE and 78 % for MAE. P value, obtained by the statistical analysis, for the models of UAE and MAE are below of significant value in all the variables, so it is understood that the parameters must be adjusted in a better way or that they are not the most influence parameters.

Using the significant regression coefficients given by the software, 3 quadratic regression equations for the extraction yield (%) were calculated, one for each extraction technique, Eq(2), Eq(3) and Eq(4).

$$\%\,\mathrm{CE} = -16.557 + 0.816187x_1 + 0.0300222x_2 - 0.00678314x_1^2 - 0.000261333x_1x_2 - 0.0000784762x_2^2 \quad (2)$$

$$\%\,\mathrm{UAE} = 3.89388 + 0.118967x_1 - 0.0692789x_2 - 0.000337662x_1^2 + 0.0000818182x_1x_2 - 0.000052571x_2^2 \quad (3)$$

$$\%\ \mathrm{MAE} = 9.82065 + 0.0505022x_1 - 0.024008x_2 - 0.00678314x_1^2 - 0.000261333x_1x_2 + 0.0000784762x_2^2 \quad (4)$$

With the aim to illustrate the main relation between the different parameters and their interactions response surface plots were drawn in Figure 1. It shows the type of interactions between all the variables and their response. Only the plot of the CE has elliptical contour, which means that there is a perfect interaction between independent variables (Zhang et al., 2014).

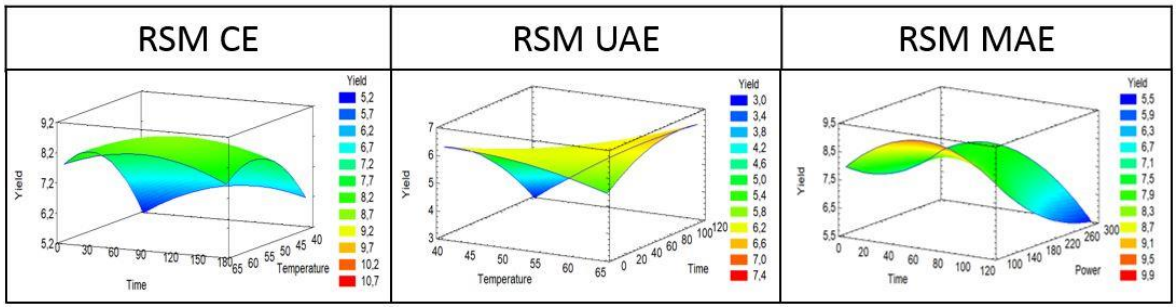

Figure 1: a) RSM plot for CE b) RSM plot for UAE c) RSM plot for MAE

3.3 Characterization of pine bark extracts

The optimal points of each extraction technique were calculated using two-level-two factor method combined with RSM, and the calculated optimal point were: for CE technique temperature was 58.26 ºC and time was 94.27 minutes; for UAE method temperature was 65 ºC and time was 94.76 minutes; and for MAE was time 62.66 minutes and power was 100 W. In these extractions different parameters were measured to get to know the antioxidant capacity as well as, total phenolic content (TPC) and total flavonoids content (TFC). Three different antioxidant capacities were measured because each one is related to a different rate. All of them are based in the reaction of a specific radical with the compounds of the extracts, and because of that, they are different. These reactions are measured by UV-VIS spectroscopy due to a colour change made during the reaction. Thus, DPPH method measured the quantity of hydrogen donors, ABTS method is based in the lost electron of the ABTS radical, and FRAP method is based in a reduction of the complex ferric ion-TPTZ (Pisoschi and Negulescu, 2011). The measured results are summarized in Table 3.

Table 3: Extraction yield and characterization of the optimized point for the extraction

Method	Extraction yield (%)	TPC (mg GAE/g dried bark extract)	TFC (mg CE/ g dried bark extract)	DPPH (mg TE /g dried bark extract)	ABTS (mg TE /g dried bark extract)	FRAP (mg TE / g dried bark extract)
CE	7.73	10.79	15.52	14.25	16.28	6.62
UAE	5.87	6.26	9.25	7.89	7.75	3.99
MAE	8.21	10.70	18.64	14.59	16.49	7.47

In general, it could be said that for almost all the measurements the obtained highest values are for MAE extracts. Total phenolic concentration in both sample, CE extracts and MAE extract, have a similar value. Nevertheless, the concentration of total phenolic for UAE extract is lower, which is accordance with the data reported by (Aspé, 2011). In the case of total flavonoids concentration, the highest value was for MAE extract followed by CE and UAE extracts. In the case of bioactivity of the extract three parameters were measured (DPPH, ABTS and FRAP) an in all of them MAE extract was the extract with the highest obtained values, and UAE extract was the one with the worst results.

4. Conclusions

In this work, we have demonstrated that the extraction techniques influence on the extraction yield, TPC, TFC and antioxidant capacity of the extraction of Larix Decidua bark. Thus, the results showed that the highest yield of extraction was for MAE as well as TPC, TFC and antioxidant capacities (DPPH, ABTS AND FRAP). This could be due to the fact that the ultrasound effect was lower that is needed to extract bioactive compounds and the long time that CE need for the extraction could degraded the compounds. However, the values for CE and MAE are not so different, but the main advantage could be the less time consumption and therefore the energy saving (Chupin et al., 2015). These results showed the potential of the Larix Decidua bark as a source of antioxidant with different possible uses in a range of application.

The optimization of the CE was carried out successfully, but for the MAE and UAE methods the optimization must be improved. However, it is clear that with the use of MAE reduces the extraction time, which could be considered as an advantage of this process.

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