

# Green high-pressure solvent extraction of health-beneficial compounds from birch bark

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## Abstract

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Pressurized hot (subcritical) water and ethanol are environmentally friendly high-diffusion solvents that can be used for fast and efficient extraction of health-beneficial compounds from agricultural- and forestry- biomass wastes. Betulin, an anti-inflammatory compound, was extracted from birch bark using ethanol at 90°C and 50 bar, resulting in quantitative yield within only 15 min of extraction. Furthermore, antioxidants in the bark were extracted at different temperatures and showed higher antioxidant activity with increasing temperature using a radical scavenging method with DPPH (2,2-diphenyl-1-picrylhydrazylhydrate) [1, 2, 3]. Investigation of how different particle sizes affect the yield of antioxidant was also studied for extraction optimization. Long slice (11 x 1cm), big bits (1 x 1 cm) and finely grinded particles were put into the extraction cells and as expected, the antioxidant activity was highest in samples of finely grinded particles. Also, pre-treatments of samples prior to extraction were performed to find out if there were chemical reactions occurring at high extraction temperature. In summary, we have developed a fast and gentle process for extraction and analysis of betulin and other health-beneficial compounds in birch bark.

## Introduction

The industrial revolution started in early 20<sup>th</sup> century in Great Britain and due to it the living condition of the human population has improved drastically. Industries of many kinds have been developed and as a result of it piles of waste have been increasing ever since then. Annually tons of wastes are produced in forestry and in agriculture industries, and at most the wastes are recycled as energy for consumption [4, 5]. Barks from different species of trees are often used as energy or as filling material in building materials. Therefore it is of our interest to develop a method which can extract beneficial components from the barks, which will not prevent the use of the bark as energy source.

Birch bark is known to have some medicinal affect against both minor and severe ailments; hence scientists have been intrigued of its chemical properties [6]. In the early 90<sup>th</sup> large resources i.e. from Russia, Finland and the U.S. have been invested to exploit the secrets of birch bark. Positive results have been revealed after years of tedious studies. The studies showed that birch bark contains different kinds of triterpenoids and their derivatives [7, 8, 9], which have shown positive effects as anti-cancer [10, 11, 12, 13], anti-HIV [14, 15, 16, 17], anti-inflammatory and anti-microbial agents [18]. Nowadays one can already find products in the market containing birch bark extract, i.e. shampoo, skincare, dental-care and hair-care products. This is due to its antioxidant capacity. Birch bark contains many different kinds of antioxidative compounds, which can be use in the products mentioned or as food additive to prevent or delay oxidation or as potential drugs.

Betulin (Lup-20(29)-ene-3,28-diol) is the major compounds in birch bark, found mostly in the outer layer of the bark, which gives its white colour and it is well scrutinized for years and showed remarkable abilities toward diseases such as cancers, HIV, and fungal infections [19, 20]. By chemical synthesis betulin can be easily transformed to betulin acid which is an even more active compound towards specific lines of cancers [21, 22, 23, 24, 25]. Experiments in

*in vitro* have already been performed therefore clinical *in vivo* experiments are in consideration. Billions of dollars are invested in birch bark's capacity as a drug ingredient; however modest effort has been put on sample preparation techniques, which is a very important step. The sample preparation methods in used are traditional ones such as "cold extraction" in many steps with organic solvents. Hence it is in our interest to develop and optimize a fast, efficient, environmentally sustainable extraction method.

Pressurised solvent extraction (PSE) is a rather new method which combines elevated temperatures and high pressure to efficiently extract interesting compounds from complex matrices. The advantage of applying high pressure in PSE is to maintain the extraction solvent as a liquid in elevated temperature. The extraction procedure becomes more efficient with increasing temperature, due to higher diffusion rates and lower viscosity and surface tension. Additionally, the solvent strength can easily be changed by simply varying the temperature, which also increases the extraction efficiency. Ethanol and water is regarded as "green" solvents and are used as extraction solvents in this study to optimize both betulin yield and antioxidative compounds in birch bark. The extraction procedure was performed at 50 bars pressures, and temperatures between 40 and 180°C, and extraction time between 5 and 15 minutes.

### Short experimental section

The extraction procedure was performed in an Accelerated Solvent Extraction (ASE-200™) system, which is trademarked by Dionex, and the size of the extraction cells used was 11 ml. For analysis, a high performance liquid chromatography (HPLC) system from Dionex, Ultimate 3000, was employed with a photodiode array detector. A Waters symmetry column (C18, 150 mm x 4,6 mm i.d, 3,5 µm), was utilized for separation of birch bark components and quantification of betulin. Data was collected and processed with a Chromeleon™ program. The antioxidant activity is determined by a free radical assay, using DPPH (2,2-Diphehyl-1-picrylhydrazylhydrate) purchased from Sigma-Aldrich Chemical Corporation (Madrid, Spain). The antioxidant activity measurements were conducted with an Ultra Violet spectrometer (UNICAM 5625 UV/VIS. Approximately 1 g of finely grinded birch bark was weighed into an 11-ml extraction cell and the extraction temperature was varied between 40 and 180°C while the pressure was kept constant at 50 bars. The extraction time was set to 5 min and the number of extraction cycles was 1, 2 or 3, whereas the flush volume was kept at 60 % (i.e. 60% of the extraction solvent in the cell was replaced in-between each extraction cycle), and with a final purging time with nitrogen of 120 s. A DPPH stock solution was daily prepared with a concentration of 0.30- 0.40 mg/ml in degassed methanol, which was later diluted with degassed methanol to a working solution (0.30 -0.40 µg/ml). Birch bark extracts were diluted 10 times with the extraction solvent (Milli-Q or ethanol) and three different volumes of the extract were taken into 4 ml vials for antioxidant activity determination. The extracts in the 4 ml vials were evaporated to dryness before 4 ml of DPPH working solution was added. The DPPH reaction was taking place in darkness at room temperature for 4 hours before measuring in UV/VIS at 516 nm. The blank sample (pure extraction solvent) was treated in the same way as the birch bark extracts.

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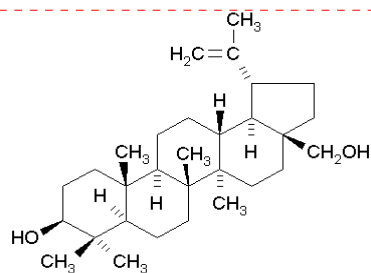
### Results and discussion

The PSE conditions were optimized to extract betulin and antioxidative components from birch bark with water and ethanol as extraction solvents. The molecular structure of betulin is as a relatively unpolar molecule (see figure 1), therefore one can expect ethanol as the better extraction solvent compare to water. The obtained results in this study verified the prediction, ethanol is indeed a more suitable solvent for betulin and zero yield of betulin was detected when using water as a solvent, at all temperatures investigated [\[26, 27\]](#).

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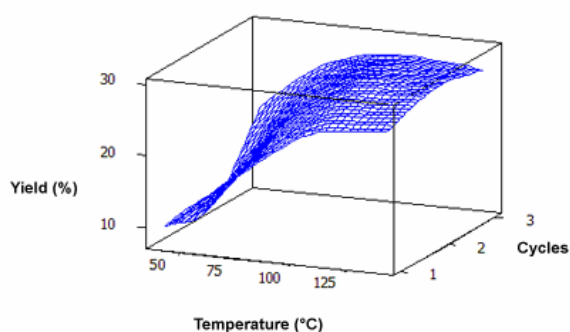
An experimental design was conducted to optimize the PSE method, in which temperature and extraction time was varied as described in the Experimental section. It turned out that the betulin yield increased up to 90°C, and then a plateau was reached (see figure 2)

Furthermore, the results showed that the numbers of extraction cycles (each cycle was 5 min) were not of significant importance compared to the temperature to achieve optimal yield of betulin. Hence, the optimized method for extracting betulin from birch bark was to use ethanol at 90°C, two extraction cycles of 5 min each, plus an initial heat-up time of 5 min, at 50 bars pressure.



**Figure 1.** The chemical structure of betulin

**Surface plot of yield vs. temperature and cycles**



**Figure 2.** The surface plot of betulin yield versus temperature and extraction cycles

### Antioxidant activity

The oxidative capacity of the antioxidative compounds in birch bark was determined with a DPPH radical scavenging method. Significant antioxidant activity was found in both ethanol and water extracts. An experimental design was applied for this study and it showed the same trend as for betulin determination, that the number of extraction cycles (i.e. extraction time 5, 10 or 15 min) did not play a significant role for the antioxidative activity. However the temperature was more important, giving higher antioxidant activity with higher temperature.

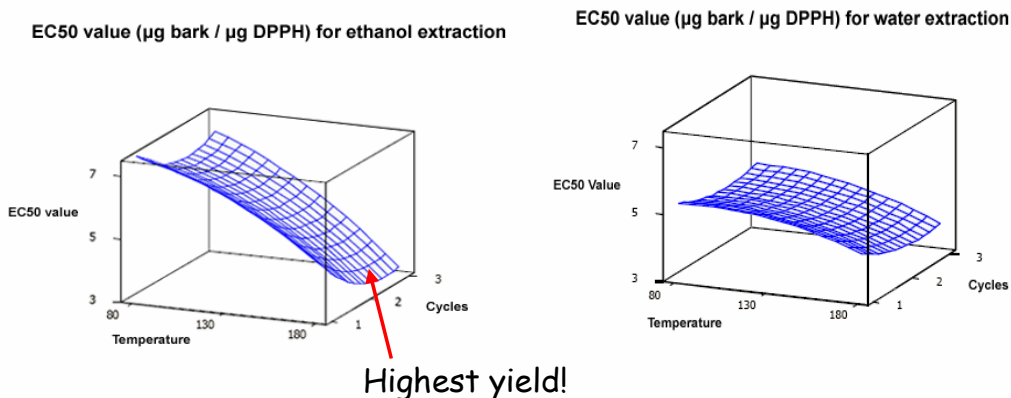
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**Figure 3.** Extraction parameters with ethanol and water as solvents for antioxidant components

The diffusion routes and mass transfer is depending on particle size, hence we studied this in samples of both outer and inner layer of birch bark (see table 1). Both outer and inner layer of birch bark was sliced in long slice (11 x 1cm), big bits (1 x 1 cm) and to finely grinded particles. The extraction parameters were set at 50 bars, 180°C and 3 extraction cycles (15 minutes) and the results showed not surprisingly, that smaller particle sizes gave improved extraction efficiency of antioxidants from the bark. Furthermore, our results also showed that the outer layer of birch bark contained significantly more quantities of antioxidative compounds than the inner layer.

**Table 1.** Effect of birch bark particle size on antioxidant activity

Experiment No (n=3)	Birch layer	Particle size	Extraction temperature (°C)	Obtained EC <sub>50</sub> values
1	Inner	Big bits	180	57
2	Inner	Finely grinded	180	37
3	Outer	long slices	180	9
4	Outer	Finely grinded	180	5

### Stability of the antioxidants

To investigate if the antioxidants in birch bark were light sensitive or not and if they degrade easily by time, a stability study was carried out with bark extracts. Samples were put uncovered in light and covered in dark during 3 months prior DPPH assay. Surprisingly, there was no significant difference in the obtained EC<sub>50</sub> values

Another interesting feature encountered in this study was the colour change at different extraction temperature. The colour tends to get darker at high temperature, from yellow at 80 °C to deep brownish red at 180 °C.

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As shown above (figure 3) the EC<sub>50</sub> value decreases with increasing extraction temperature. However, is this due to higher extraction yield of antioxidants or due to some chemical reaction (e.g. hydrolysis of polyphenolic glycosides) during the extraction that results into higher total antioxidant activity of the sample? In an attempt to figure this out, birch bark samples were pre-treated in water to 180 °C and cooled to ambient temperature during 4 hours prior extraction at 80 °C respective 180 °C. The antioxidant activity was then determined by DPPH and resulted in a higher antioxidant activity for pre-treated samples at 180 extraction temperature (see table 2).

**Table 2.** Pre-treatment of birch bark in water prior to extraction at different temperatures

Experiment No (n=3)	Pre-treatment conditions	Extraction temperature (°C)	Obtained EC <sub>50</sub> values
1	180°C in MQ at 50 bars	80	8
2	180°C in MQ at 50 bars	180	2
3	4 hrs in MQ	80	93
4	4 hrs in MQ	180	16

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Unfortunately, the results are not conclusive, because an increasing antioxidant activity in samples that have been pre-treated with hot water could be a result of a “pre-extraction” of the antioxidants from the sample matrix. Hence, more experiments are at this moment going on to find out if the chemical composition has changed with higher extraction temperature to see if some molecules have degraded and thereby become more active. What we know today, is that the best method for extracting antioxidants from birch bark is to use the highest temperature tested, i.e. 180°C, with either ethanol or water as extraction solvent, for 2 cycles of 5 min each, plus an initial heat-up time of 8 min (or whatever time needed to accomplish the set temperature inside the extraction vessel), at 50 bars pressure.

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### Conclusions

In summary, the developed extraction methods are simple, fast, efficient and environmentally friendly using solvents as ethanol and water to extract health beneficial compounds from birch bark. Our results show that the extraction yield of betulin and the antioxidative components increases with increasing temperature, although reaching a plateau for betulin at around 90°C. The choice of birch bark layer is crucial in the extraction procedure, since the outer layer contains more of betulin and also compounds with high antioxidant capacity. Experimental attempts have been done to investigate if any chemical reactions occur at high temperature that could potentially increase the antioxidant activity of the compounds in the bark. Additionally, more experiments with LC-MS analyse or some kinetic studies are needed to verify if any chemical reaction transpire at high temperature.

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