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## Microwave Assisted Extraction of betulin from birch outer bark†

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In this communication, we report a new method for the isolation of betulin, a triterpenoid with interesting pharmacological activities, from birch outer bark. Remarkably high purity betulin raw extracts were obtained, ca. 95%, through microwave assisted extraction with limonene. This saves time and energy when compared to the conventional methods.

Plants exhibit an outstanding diversity of complex biologically active molecules, which are difficult, or even impossible, to synthesise chemically. These compounds, which constitute the so called plant extractives, include some which have long been used as therapeutic agents and thus are increasingly inspiring the design of new drugs.<sup>1-4</sup> Plant extractives are normally isolated using simple solvent extraction methods.<sup>5-7</sup> However, the compositional variability and the complexity of the obtained extracts together with the poor performance of the extraction method and tight legislation/safety concerns and restrictions, hinder their straightforward application.<sup>3, 8</sup> Upon extraction, additional purification steps are usually required to assure satisfactory purity levels. These observations have prompted the development of alternative extraction methods for targeted compounds.<sup>5-7</sup>

Betulin (lup-20-(29)-ene-3,28-diol or betulinol, Fig. 1) is a naturally occurring lupane triterpenoid particularly abundant in the outer bark of birch species (up to ca. 25 wt%).<sup>9-11</sup> Crystalline deposits of betulin rapidly accumulate in peridermal cells during spring and nearly fill the intracellular space in summer.<sup>12, 13</sup> The therapeutic and the pharmacological properties of betulin (and its derivatives) have intensively been investigated and properly reviewed in the last decade.<sup>11, 14</sup> In resume, betulin exhibits a wide spectrum of biological activities, such as antitumor, anti-HIV, antibacterial and anti-inflammatory properties. In addition, this triterpenoid can be easily converted in high yields to betulinic acid,<sup>15</sup> a derivative currently in clinical trials<sup>16, 17</sup> that shows strong biological activity against HIV and acts as a specific inducer of apoptosis in cancer cells.<sup>11, 14</sup>

Betulin is a non-structural element of the plant cell wall (*i.e.* not covalently bonded), thus it is usually isolated from birch outer bark by conventional Soxhlet extraction using organic solvents such as methanol, ethanol, dichloromethane or chloroform.<sup>9, 18-21</sup> Complete removal takes several hours, usually 3 to 8, and requires solvent reflux, meaning time and energy consumptions.

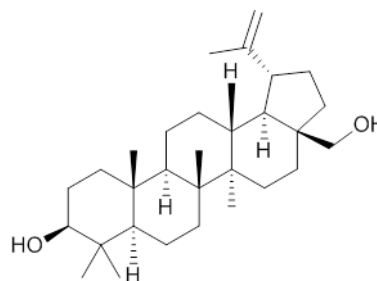


Fig. 1 Molecular structure of betulin.

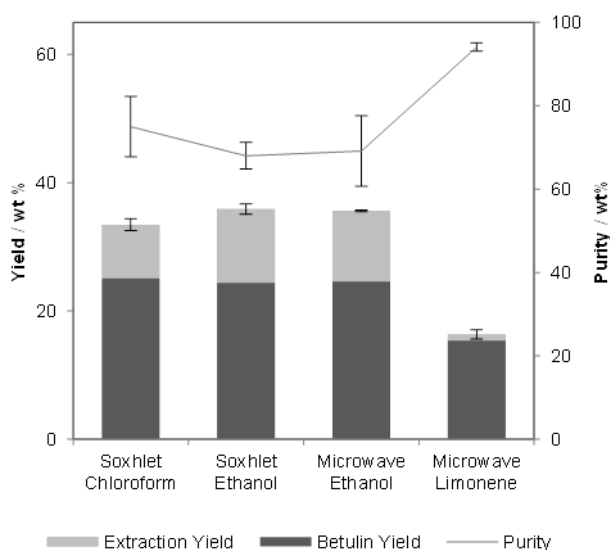
The purity level of betulin in these raw extracts is low, typically ca. 80%.<sup>9, 11</sup> With the purpose of reaching higher purity levels, alternative extraction techniques and/or solvents have been also tested and include sublimation,<sup>18, 22</sup> supercritical carbon dioxide extraction,<sup>23</sup> ultrasound assisted extraction,<sup>24</sup> ionic liquid extraction<sup>25</sup> and microwave assisted extraction,<sup>25</sup> as well as combination of the aforementioned methods. Even so, betulin extraction is generally followed by one or more purification steps such as crystallisation and/or chromatography.<sup>18, 19, 25, 26</sup>

In recent decades, microwave assisted extraction has drawn significant attention, particularly in medicinal plant research since it allows the isolation of plant extractives in high yields, within a short period and with low energy consumption.<sup>5-7, 27</sup> Microwaves induce a rapid, efficient and homogeneous heating within the bulk of the extraction medium (solvent and plant matrix), resulting in internal superheating of the water typically present within the plant matrix. This promotes efficient cell disruption facilitating desorption and recovery of the plant extractives. In addition, the presence of molecules with permanent dipoles (*e.g.* water) in the extraction medium also explains why even microwave transparent solvents can be efficiently applied in this technique.<sup>5, 6</sup>

In this study, the ability of ethanol and limonene ((*R*)-(+)-limonene) to extract betulin from birch outer bark (milled to < 1 mm) through microwave assisted extraction was evaluated. Limonene, a commercially available and FDA approved terpene, which belongs to the same chemical class of betulin, seemed to be a rational selection as an alternative solvent. Despite widely used at industrial levels as a flavour/fragrance additive and as degreaser, few studies have reported the use of limonene as solvent, *e.g.* the Soxhlet extraction of terpenoids from plants<sup>28</sup>

and the microwave assisted extractions of fatty acids<sup>29</sup>. Notwithstanding that limonene is a moderately microwave absorbing solvent (dielectric constant,  $\epsilon = 2.3$ ),<sup>29</sup> the presence of low amounts of water in the bark matrix ( $1.8 \pm 0.4$  wt%) promotes efficient microwave-heating.

The microwave assisted extractions were carried out only during 30 min at isothermal conditions, 150°C, using a bark to solvent weight ratio of 5 wt%. Immediately after the microwave assisted extractions, the bark was removed by filtration (0.45  $\mu$ m, nylon). For recovering the raw extracts, ethanol was eliminated from the ensuing filtrate by solvent evaporation. When using limonene, the raw extracts were precipitated using an anti-solvent, namely cyclohexane or ethyl formate (solvent:anti-solvent volume ratio, 1:2; 4 °C, 12 hours), then recovered simply by filtration (0.45  $\mu$ m, nylon). The temperature, pressure and power curves of microwave assisted extraction with limonene are depicted in Fig. ESI1. The results obtained using microwave assisted extraction were compared to those obtained by Soxhlet extraction using either ethanol or chloroform as solvents, during 8 hours - a sufficient time period for complete removal of the birch outer bark extractives. After the extraction the samples were recovered by solvent evaporation. The extraction yields and betulin purity levels for all samples were determined by weighing the dried extracts and by GC-MS analysis, respectively (Fig. 2).



**Fig. 2** Extraction yields and purity of the betulin raw extracts obtained by soxhlet and microwave assisted extraction. Betulin yields were calculated accounting the extractions yields and their purities.



**Fig. 3** Birch outer bark extracts obtained by Soxhlet and microwave assisted extractions. The extraction method and the used solvent are indicated in the figure.

The extractable fraction of birch outer bark was *ca.* 35 wt%, as determined by Soxhlet extraction using ethanol or chloroform (Fig. 2). However, when accounting for the purity of these extracts, the amount of betulin corresponds to *ca.* 25 % of the initial bark weight. This value equals that typically found in birch outer bark.<sup>9-11</sup> Similar extraction yields and purities were obtained after half an hour of microwave assisted extraction with ethanol (Fig. 2). This demonstrates that by using microwave assisted extraction, time and energy savings can be promptly attained when compared to the conventional Soxhlet extractions.

All the above mentioned betulin raw extracts displayed limited purities, *i.e.* below 75 % (Fig. 2). This is due to the low selectivity of the extraction solvents towards the solute. On the contrary, the raw extracts obtained by microwave assisted extraction with limonene showed a remarkably high betulin content, *ca.* 95 wt%. In this case the precipitated raw extracts accounted for 16.3 % of the initial bark weight (Fig. 2). Further improvement of the recovery step is envisaged since limonene was able to dissolve 24.4 % of the initial bark weight. This suggests that limonene dissolved virtually all the available betulin. The high purity of the obtained extracts is highlighted by their clear white colour, whereas the raw betulin extracts obtained either with ethanol or chloroform, regardless of the extraction method, presented a light brown or pale yellow colour, respectively (Fig. 3).

GC-MS analyses confirmed that the raw extracts contain, in addition to betulin, minor amounts of other compounds naturally present in birch outer bark (Table 1, Fig. ESI2).<sup>10, 11</sup> Triterpenoid contaminants, in particular lupeol, were predominantly detected in the betulin raw extracts obtained with ethanol and chloroform, whereas almost undetected in those obtained with limonene. Similarly, other compounds such mono- and disaccharides, sterols, long chain alcohols, fatty acids, hydroxycinnamic acids and glycerol were also detected (Table1, Fig. ESI2), although once more almost absent in the extracts obtained with limonene. These observations constitute a further demonstration of the high selectivity of limonene towards betulin, notwithstanding of any minor contribution of the antisolvent to the purity of the extracts.

The betulin raw extracts obtained with limonene through microwave assisted extraction were characterized by <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance (NMR) spectroscopy and compared to those of standard betulin (98%, Extrasynthese, France). The

obtained spectra were virtually similar, supporting the high purity of the raw extracts (Fig. ESI3).

**Table 1** Main components detected in birch outer bark extracts.

Chemical identification was done by comparing mass spectra with the equipment mass spectral library (NIST mass spectral library 2005) and their characteristic retention times and fragmentation profiles with published data.

	Amount (Standard deviation) / wt %			
	Microwave Limonene	Microwave Ethanol	Soxhlet Chloroform	Soxhlet Ethanol
Betulin	94,1 (1.0)	69,1 (8.4)	75,0 (7.3)	68,0 (3,2)
Lupeol	0,5 (0.1)	12,3 (1.6)	11,0 (0.5)	9,3 (0,4)
Betulinic acid	3,2 (0.9)	3,1 (0.4)	2,7 (0.3)	2,4 (0,3)
Erythrodiol	0,2 (0.1)	0,4 (0.1)	0,3 (0.0)	0,3 (0,1)
Betulin Aldehyde	0,1 (0.0)	0,8 (0.1)	0,6 (0.0)	0,5 (0,1)
Others <sup>a</sup>	1,9	14,3	10,4	19,5

<sup>a</sup> includes variable amounts of mono- and disaccharides, sterols, long chain alcohols, fatty acids, hydroxycinnamic acids and glycerol. The amount of these compounds was determined as the difference to 100%.

The data herein presented demonstrate that microwave assisted extraction is a highly efficient technique to rapidly isolate birch bark extractives. In the method herein reported limonene can be recovered simply by evaporation of the anti-solvent used for promoting the precipitation of the extractives. It can be reused, even if containing minor amounts of non-precipitated betulin. To the best of our knowledge, up to date, a single study has focussed on microwave assisted extraction of betulin; however the good performance of the method is hampered by considerable degradation of the solvent.<sup>25</sup>

The findings herein reported constitute an important advance in the isolation of highly pure betulin raw extracts from birch outer bark. It combines the benefits of the microwave assisted extraction technique with the high selectivity of limonene towards betulin. Further work is envisaged, namely the optimisation of the operation conditions through factorial design of experiments. Birch bark constitutes a cheap biomass residue readily available in high quantities, especially in paper pulping industries, where it is burned for energy production.<sup>11</sup> Therefore when considering the limited demand of betulin, birch bark can be regarded as virtually unlimited source of this triterpenoid. For instance, from an average paper mill *ca.* 1800 tons of betulin could be produced annually. These quantities largely exceed the current demands and those of prospective markets.<sup>11</sup> Birch outer bark constitutes also a unique source of suberin,<sup>30</sup> a value-added natural biopolyester that is often regarded as a source of unique building blocks for the development of innovative materials. The integrated extraction of suberin and betulin from birch bark would certainly boost the value of this residue. Notably, the developed knowledge should encourage the application of this process to the selective extraction of other triterpenoids from numerous biomass residues.

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# Microwave Assisted Extraction of <sup>30</sup> betulin from birch outer bark

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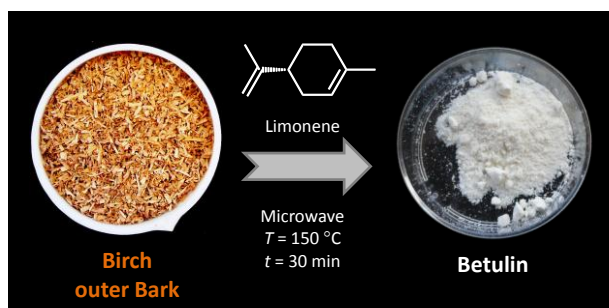
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A novel microwave assisted extraction method for  
25 isolating betulin, a biologically active triterpenoid,  
from birch outer bark. Remarkably, the raw extracts  
obtained using limonene as solvent show  
extraordinarily high purity.