

Recovery of betulinic acid from plane tree

(Platanus acerifolia L.)

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Abstract

Betulinic acid (3 β , hydroxy-lup-20(29)-en-28-oic acid) is a bioactive triterpenic acid which was identified in various botanical sources and in considerable amounts in the bark of plane tree (*Platanus acerifolia* L.). In this work, the recovery of betulinic acid from plane tree bark was studied using different liquid solvent based extraction methods, namely Solid-Liquid Extraction (SLE), Ultrasound Assisted Extraction (UAE) and Pressurized Liquid Extraction (PLE). Furthermore, preliminary studies of the Supercritical Fluid Extraction (SFE) of plane tree bark are also reported.

The liquid solvent based extraction techniques (SLE, UAE and PLE) were carried out using ethanol and ethyl acetate, and produced a recovery of betulinic acid in the range 10-15 mg per g of bark, with concentrations around 25-35 % mass. A betulinic acid enrichment in the ethanolic extracts was possible by means of a simple precipitation step adding water. The precipitate contained 42-46 % mass of betulinic acid and high

1 recovery (> 95%). Increasing the extraction temperature, by means of the PLE assays,
2 has not resulted in an improvement of betulinic acid recovery.
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4 The preliminary SFE assays produced lower recoveries of betulinic acid (0.5-8 mg/g)
5 with respect to liquid extraction. The addition of ethanol as cosolvent produced a
6 significant improvement of both betulinic acid recovery and concentration in the SFE
7 extract.
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16 **Keywords:** supercritical fluid extraction; pressurized liquid extraction; ultrasound
17 assisted extraction; betulinic acid; plane tree bark; *Platanus acerifolia*.
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1. Introduction

Triterpenic acids are secondary plant metabolites that are widespread in plants, mainly located in the peel, leave and stem bark [1]. They are part of the chemical family of isoprenoids, owning polycyclic structures of thirty carbon atoms, and presenting very low solubility in water and hydrophilic solvents. On the other hand, their solubility in organic solvents such us acetone or methanol has been demonstrated to be moderately high [2].

Betulinic acid (3 β , hydroxy-lup-20(29)-en-28-oic acid) is a triterpenic acid which can be isolated from various botanical sources, including clove (*Syzygium aromaticum*), Lamiaceae herbs such as rosemary (*Rosmarinus officinalis*) and java tea (*Orthosiphon stamineus*), and the bark of several betula species (birch trees), eucalyptus (*Eucalyptus globulus*) and plane (*Platanus acerifolia*) trees [1, 3-8].

Betulinic acid as well as its derivatives, have demonstrated a wide range of biological activities, including anti HIV-1 activity [9], anti-inflammatory activity [10], antimalarial activity [11], anticancer and apoptotic activity [12, 13]. Additionally, it has been demonstrated that some changes in betulinic acid structure can lead to significant differences in its anticancer and antiproliferative activity [14, 15].

The presence of betulinic acid at concentrations up to 3 % (30 mg/g) in the external dried bark of plane tree (*Platanus acerifolia* L.) was previously reported [1, 16, 17]. These works focused in the extraction of betulinic acid from the bark of plane tree through conventional solid-liquid extraction with methanol, chloroform and heptane. Nevertheless, to our knowledge, the extraction of phytochemicals present in this botanical source has not been thoroughly studied yet.

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Novel liquid solvent based extraction methods include the assistance of solid-liquid extraction using ultrasounds (UAE), and the use of high extraction temperatures by increasing also pressure to maintain the solvent in liquid state (PLE).

The use of UAE to recover triterpenic acids from different plant matrix has been recently studied [18-20] and has proved to present several advantages in comparison with conventional solid-liquid extraction. These advantages include reduction of the amount of solvent required, time and temperature, which represents an important factor when extracting thermolabile compounds [21]. Ultrasonic cavitation enhances mass transfer through its capability to facilitate hydrating and swelling of vegetal tissues as well as diffusion and osmotic processes [22].

Also PLE to recover triterpenic acids from different botanical sources has been previously reported [19, 23]. PLE uses high pressures in order to remain solvents in liquid state beyond their normal boiling point. The combination of high pressures and high temperatures enhances mass transfer, thus facilitating the extraction process. It has demonstrated several advantages in comparison to traditional extraction procedures, mainly the decrease of both time and amount of solvent. However, the lack of industrial scale pressurized liquid extraction equipments, lead to a moderate application of this technique.

Supercritical fluid extraction (SFE) using carbon dioxide (CO₂) was also utilized to recover triterpenoid acids from different plant matrix, as reported by Domingues et al. [6-8], Felföldi-Gáva et al. (2009) [24], Melo et al. (2012) [25] and Zhao (2011) [26] among others. Due to its low polarity, supercritical CO₂ has shown a moderate capacity to dissolve this type of compounds and thus, the use of ethanol as cosolvent has been employed as a suitable alternative to increase triterpenic acid recovery. An appropriate

1 combination of pressure and ethanol as cosolvent may increase the yield of triterpenic
2 acids profusely [7].
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4 In this paper different advanced extraction techniques (UAE, PLE and SFE) and
5 conventional solid-liquid extraction (SLE) are studied and compared, with the target of
6 recovering betulinic acid from the bark of *Platanus acerifolia* L. Different GRAS
7 (General Recognized as Safe) solvents were utilized (ethanol, ethyl acetate and SCCO₂)
8 and different process conditions were investigated.
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19 **2. Material and Methods**

20 **2.1 Chemicals**

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26 Ethanol Absolute (99.5% purity), Ethyl acetate (99%, purity) was purchased
27 from Panreac (Barcelona, Spain). CO₂ was used as the supercritical solvent with a
28 purity of 99.9% produced by Carbueros Metalicos, S.A. (Madrid, Spain). Betulinic Acid
29 reference Standar was purchased from Extrashyntesse (Genay, Cedex, France).
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39 **2.2 Analysis**

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41 Quantification of betulinic acid (BA) in the extracts was performed by HPLC
42 Agilent 1200 series from Agilent Technologies Inc. (Santa Clara, California, USA)
43 according to a method previously described [19] with some modifications. Briefly,
44 separation was carried out using a C-18 reverse phase column (250 x 4.6 mm, 5 μm),
45 with a mobile phase consisting of HPLC grade acetonitrile-MilliQ water-phosphoric
46 acid (80:20:0.04, v/v/v). Elution was performed isocratically at a flow rate of 1 ml/min,
47 at 25 °C, and with a total analysis time of 30 min. Injection volume was 10 μL and
48 spectral data was recorded at 210 nm. Data analysis was performed by ChemStation
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1 version B.04.03. Samples were prepared using methanol at 0.7 mg/ml. Calibration
2 curves of BA were constructed with reference standard.
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7 **2.3 Preparation of Sample**

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9 4 kg of plane tree bark (*Platanus acerifolia* L.) were collected in the Campus of
10 Universidad Autónoma de Madrid (Madrid, Spain) and were air dried at ambient
11 temperature for 72 h. The final content of water in the dried sample was determined in
12 an oven at 105°C (48 h) and resulted 9.5 % mass. The bark was ground in a grind
13 Premil 250 (Lleal S.A., Barcelona, Spain) to a mean particles size of 500 µm and
14 packed and stored at room temperature until utilization.
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27 **2.4. Extraction techniques**

28 **2.4.1 Solid-Liquid Extraction (SLE)**

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32 35 g of ground *Platanus* bark were extracted with 350 mL of solvent (ethanol or
33 ethyl acetate) at 45°C using a magnetic stirrer. Extraction time was 1.5 h. The infusion
34 was filtrated in a vacuum flask with a Büchner funnel and the sifted material was
35 washed with 50 mL of solvent. The liquid phase was concentrated at low temperature
36 (35°C) in a rotavapor (VWR from IKA Works GmbH & Co., Staufen, Germany).
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46 In order to produce a triterpenic acid enrichment, 35 g of raw material were
47 extracted with 350 mL of ethanol, as aforementioned, and the ethanol was removed in
48 rotavapor until 1/3 of the initial volume. Then, an equal volume of deionized water was
49 added, and the mixture was stored at room temperature for 30 min until a white
50 precipitated was formed. The precipitated was collected by filtration and was dried in a
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1 freeze dryer from Labconco Corporation (Missouri, USA). The liquid mixture
2 (water/ethanol) was concentrated in rotavapor and freeze-dried.
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4 Extractions were carried out by duplicate and all samples were stored under
5 refrigeration until they were analyzed.
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8 9 10 11 **2.4.2 Ultrasound Assisted Extraction (UAE)**

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14 35 g of ground Platanus bark with the corresponding solvent (ethanol or ethyl
15 acetate) in a ratio 1:5 (bark:solvent) were submitted to ultrasounds for 15 min using a
16 1/2" diameter disruptor horn probe at 70% amplitude (maximum power output of 400
17 Watts at 60 Hz) (Branson Digital Sonifier, Branson Ultrasonics, model 250; Danbury,
18 USA) maintaining temperature at 45°C. Sonication at the desired amplitude level was
19 started once the set temperature was reached. The ultrasound probe was submerged to a
20 depth of 25 mm in the sample. The input range of the selected variables was determined
21 by preliminary experiments and the UAE conditions were selected on the basis of
22 previous studies reported in the literature [27-30].
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36 Extractions were carried out by duplicate. After sonication, the samples were
37 filtrated and dried in rotavapor. In the case of ethanol experiments, the same procedure
38 described in the case of SLE was applied after UAE in order to attain a triterpenic acid
39 enrichment. All samples were stored under refrigeration until they were analyzed.
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48 **2.4.3 Supercritical Fluid Extraction (SFE)**

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51 Extractions were carried out using a pilot plant supercritical fluid extractor from
52 Thar Technology (model SF2000; Pittsburgh, Pensilvania, USA) comprising a 2 L
53 cylindrical extraction vessel (internal diameter = 0.07 m; height = 0.388 m) and two
54 different separators, with 0.5 L capacity each one, independent temperature control (± 2
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1 K) and pressure (± 0.1 MPa). The extraction device also includes a recirculation system
2 where CO₂ is condensed and pumped up to the desired extraction pressure. A detail
3 explanation of the experimental SFE device employed can be found elsewhere [31].
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7 For each experiment, the extraction vessel was packed with 0.57 kg of ground
8 plane tree bark (apparent density = 381.7 kg/m³). The extraction conditions are given in
9 Table 4, and were performed at 313 K, pressure range of 25-50 MPa and with an
10 upwards CO₂ flow rate of 50 g/min. The overall extraction time was set to 4 h.
11 Extractions 1 and 2 (see Table 4) were carried out in two different steps: the first step
12 (1.5 h) at 25 MPa and without cosolvent and the second step (2.5 h) at 30 MPa and
13 using, respectively, 10% and 20% of ethanol cosolvent. Extraction conditions were
14 selected on the basis of previous studies reported in the literature [6].
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29 **2.4.4 Pressurized Liquid Extraction (PLE)**

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31 Extractions were carried out in an Accelerated Solvent Extraction System ASE
32 350 from Dionex Corporation (Sunnyvale, CA, USA) equipped with a solvent controller
33 unit. Extractions were performed with two different liquid solvents (ethanol and ethyl
34 acetate) at three different extraction temperatures (100, 150 and 200°C) using 1 g of
35 solid sample and 1 g of sea sand as a sandwich. The consumption of solvent during
36 extraction (amount of solvent required to fill the extraction cell) was around 10 ml.
37 Extraction conditions were selected on the basis of previous studies reported in the
38 literature [32]. All extractions were made by duplicate. Extracts were dried using a
39 rotavapor and were stored under refrigeration until analysis.
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56 **3. Results and discussion**

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Tables 1 to 3 present, respectively, the results obtained in the SLE, UAE and PLE experiments. The results reported in the tables include the extraction yield (mass of extract / mass of dried bark), the concentration of betulinic acid (BA) in the extract (% mass) and its recovery (mg BA / g of dried bark).

The extracts obtained by SLE using ethanol or ethyl acetate present similar yield and BA concentration and thus, similar BA recovery. Fractionation of the ethanolic extract by adding water resulted in a precipitate with 41.48 % mass of BA, and around 96 % of BA recovery in the solid phase.

The assistance of solid-liquid extraction with ultrasounds (UAE temperature was maintained equal to the SLE temperature) produced a slight increase of BA recovery when ethanol is employed ($\approx 10\%$), while significant higher recovery was obtained with ethyl acetate (14.74 vs. 10.41 mg/g, i.e. an increase close to 40%). Nevertheless, the concentration of BA in the ethyl acetate UAE extract is lower than that obtained in the SLE extract (27.76 vs. 32.63 % mass). Moreover, it has to be taken into account that UAE consumed half the amount of solvent (solvent/bark ratio = 5) and lower extraction time (15 min) in comparison with SLE.

The fractionation procedure applied to the ethanolic UAE extract produced similar result than in the case of the ethanolic SLE extract: a BA enriched precipitate was obtained (46.21 % mass) with 98% recovery (slight loss of BA in the supernatant aqueous phase).

The results obtained in the PLE of plane tree bark are given in Table 3. The effect of increasing temperature in PLE (100, 150 and 200°C) is producing higher yields but lower BA concentrations in the extract. That is, higher extraction temperatures favor the extraction of compounds other than BA, which was almost exhausted from the raw material, as can be deduced from the similar recoveries obtained despite the extraction

1 temperature applied. Accordingly, in SLE (extraction temperature of 45°C) the lowest
2 yields and the highest BA concentrations were obtained, maintaining almost the same
3 (slightly lower) BA recovery. These conclusions hold for both ethanol and ethyl acetate
4 solvents, as illustrated in Figure 1. Although the optimization of solvent consumption
5 was not a target of this work, it is evident that the UAE extracts were obtained using
6 half the amount of the solvent employed in the SLE and PLE, and higher BA
7 concentrations and similar BA recovery were attained. Thus, no advantage can be
8 established in favor of using PLE instead of SLE or UAE, particularly if extracts with
9 high betulinic acid content are target.

21 Tables 4 present the results obtained from the SFE of plane tree bark.
22 Extractions 1 and 2 were carried out in two steps: the first step was carried out at 25
23 MPa and 40°C, without using cosolvent, while the second step was performed at 30
24 MPa, 40°C and using 10% ethanol (Ext. 1) or 20% ethanol (Ext. 2) as cosolvent. The
25 two-step approach was accomplished expecting that low amounts of BA were extracted
26 in the first step, and high concentration of the acid may possibly be achieved in the
27 extract produced in the second step due to the addition of ethanol as CO₂ cosolvent. Ext.
28 3 in Table 4 was carried out at higher pressure (50 MPa) and without cosolvent.
29 Additionally, fractionation of the extract using the cascade decompression system was
30 employed with the objective of producing a sample with high concentration of BA in
31 the first separator.

48 The low yield obtained in the first step of Extractions 1 and 2 resulted in just a
49 slight increase of BA concentration in the second fraction of the two-step procedure. On
50 the other side, the on-line fractionation applied when pure CO₂ was utilized (Ext. 3 in
51 Table 4) produced a significant concentration of BA in the extract precipitated in the
52 first separator. Although this fractionation alternative can produce a sample with 10.85

1 % mass of BA, the use of ethanol as cosolvent is an important variable to be
2 investigated in order to maximize SFE of betulinic acid from plane tree bark (BA %
3 mass was 15 and 18 % when, respectively, 10 and 20 % ethanol cosolvent was utilized).
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5 The CO₂/feed ratio employed in this work was 21 kg/kg, similar to the value utilized by
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7 Dominguez et al. [6] (27 kg/kg) in the SFE of triterpenic acids from eucalyptus bark.
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9 Despite the % cosolvent was proven to be a very important variable in the SFE of plane
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11 bark, the CO₂/feed ratio is also a variable that should be investigated and optimized.
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14 In the range of SFE experimental conditions investigated all liquid solvent based
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16 extraction techniques (SLE, UEA and PLE) produced higher recovery of BA (10-15
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18 mg/g) than those obtained with SFE (ca. 8 mg/g when using ethanol as CO₂ cosolvent).
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21 With respect to the concentration of BA in the extract, ethanol and ethyl lactate
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23 produced extracts with values up to 25-35 % mass, higher than the maximum obtained
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25 in SFE (18 % mass).
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31 Figure 2 shows a comparison between the extraction methods using liquid
32 ethanol (SLE, UAE and PLE) and the SFE using ethanol as CO₂ cosolvent. Figure 2(a)
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34 indicate that all liquid solvent based methods produce high BA recovery (\approx 12 mg BA /
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36 g bark) while very low BA recovery is obtained by SFE with pure CO₂ despite the high
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38 extraction pressure applied (50 MPa). The addition of 10-20% ethanol significantly
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40 increases BA recovery up to values close to those obtained by liquid ethanol extraction.
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42 Figure 2(b) depicts the concentration of BA (% mass) obtained in the different extracts.
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44 It can be observed in the figure how the addition of ethanol cosolvent in the SFE results
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46 in an increase of BA % mass in the extract. Yet, UAE extract present around a two-fold
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48 increase of BA concentration in comparison with the SFE extracts. Finally, Figure 2(c)
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50 compares the ethanol consumed by each technological approach. Although the
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52 consumption of ethanol was not optimized in this work, it can be concluded from the
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1 figure that lower amount of ethanol is required in SFE technology per unit of mass of
2 target BA recovery.
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7 **4. Conclusions**

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9 Considering the results obtained in this work, it can be concluded that the plane
10 tree bark extract with higher concentration of betulinic acid was obtained by ethanol
11 extraction assisted with ultrasounds and followed by a simple pre-fractionation step
12 using water. This approach produced an extract with 46.21 % mass of betulinic acid and
13 2.7 % yield. Furthermore, ethyl acetate UAE can produce almost a two fold increase of
14 extraction yield (5.31 %) with ca. 28 % mass of betulinic acid in the extract.
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24 The preliminary SFE accomplished in this work, permit to presume that the use
25 of ethanol as CO₂ cosolvent has the most significant effect on the extraction of betulinic
26 acid from plane tree bark. In comparison with SLE, UAE and PLE, 20 % ethanol
27 cosolvent resulted in high yield (4.34 %), good concentration of betulinic acid in the
28 extract (18.30 % mass) and almost one third of ethanol consumption. Further
29 investigation to optimize SFE conditions and attain higher betulinic acid recovery is
30 necessary.
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Table 1. Solid-liquid extraction (SLE) of plane tree bark at 45°C. BA: betulinic acid.

Solvent		Yield (%)	BA concentration (% mass in the extract)	BA recovery (mg / g dry matter)
Ethanol		3.69	29.51	10.89
Ethanol	Precipitate	2.22	41.48	9.21
	Supernatant	0.93	3.10	0.29
Ethyl Acetate		3.19	32.63	10.41

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Table 2. Pressurized liquid extraction (PLE) of plane tree bark at 100, 150 and 200°C.

BA: betulinic acid.

Solvent	T (°C)	Yield (%)	BA concentration (% mass in the extract)	BA recovery (mg / g dry matter)
Ethanol	100	4.88	25.03	12.21
	150	6.88	18.52	12.74
	200	11.92	10.89	12.98
Ethyl Acetate	100	4.39	26.69	11.72
	150	4.70	21.79	10.24
	200	6.21	18.87	11.72

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Table 3. Ultrasound assisted extraction (UAE) of plane tree bark at 45°C. BA: betulinic acid.

Solvent		Yield (%)	BA concentration (% mass in the extract)	BA recovery (mg / g dry matter)
Ethanol		3.52	33.82	11.90
Ethanol	Precipitate	2.70	46.21	12.48
	Supernatant	0.98	1.52	0.15
Ethyl Acetate		5.31	27.76	14.74

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Table 4. SC-CO₂ extraction of plane tree bark at 40°C. BA: betulinic acid.

	Ext 1		Ext 2		Ext 3
	Step 1	Step 2	Step1	Step 2	
Pressure (MPa)	25	30	25	30	50
time (h)	1.5	2.5	1.5	2.5	4
Ethanol cosolvent (% mass)	0	10	0	20	0
Extraction yield (%)	0.66	4.94	0.69	4.34	0.35 (S1) 0.74 (S2)
BA concentration (% mass in the extract)	0.23	14.99	2.36	18.30	10.85 (S1) 2.18 (S2)
BA recovery (mg / g dry matter)	0.02	7.41	0.16	7.94	0.38 (S1) 0.16 (S2)

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List of Figures

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4 **Figure 1.** Effect of temperature on the extraction of betulinic acid from *Platanus*
5 *acerifolia* bark: % mass betulinic acid in the extract (black columns), extraction yield
6 (white columns) and betulinic acid recovery (grey columns). SLE: 45°C; PLE: 100, 150
7 and 200°C. (a) Ethanol; (b) ethyl acetate solvent.
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12 **Figure 2.** Comparison between the extraction methods using liquid ethanol (SLE, UAE
13 and PLE) and SFE with 0, 10 and 20 % ethanol cosolvent. (a) BA recovery; (b) BA
14 concentration; (c) ethanol consumption.
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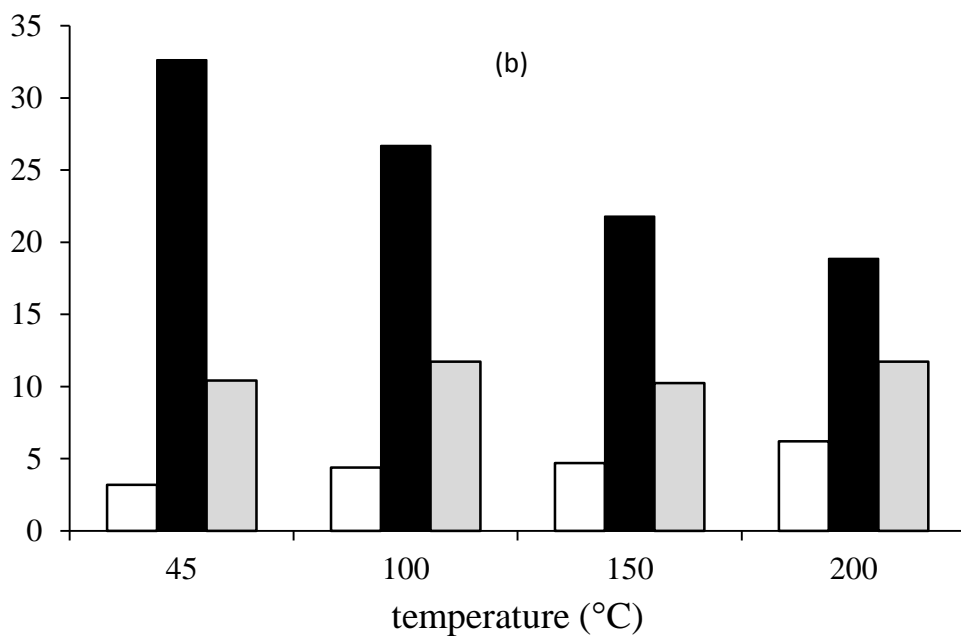
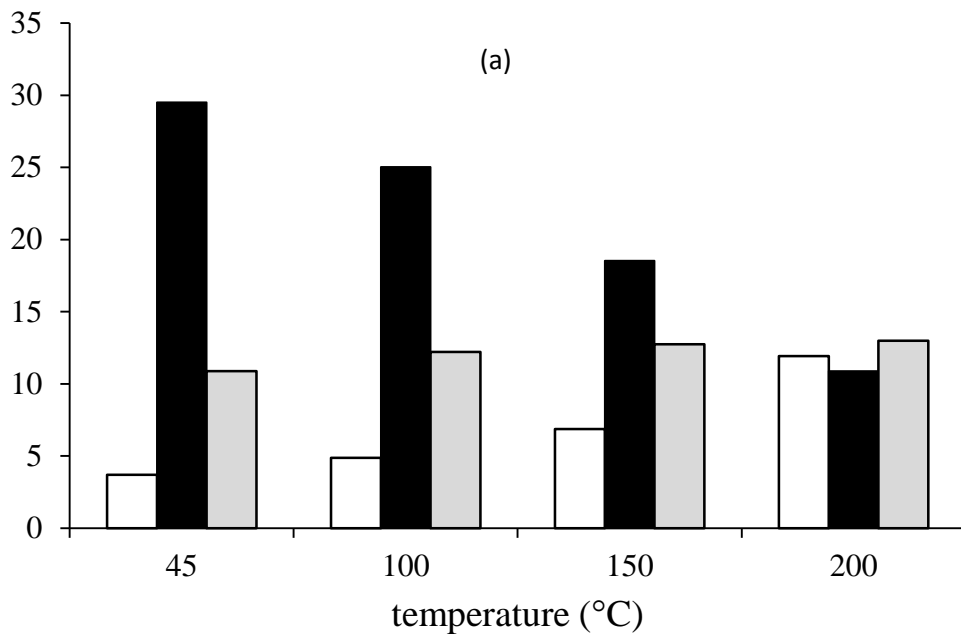


Figure 1. Effect of temperature on the extraction of betulinic acid from *Platanus acerifolia* bark; **black columns:** concentration of betulinic acid in the extract (% mass), **white columns:** extraction yield (mass extracted / mass bark x 100), **grey columns:** betulinic acid recovery (mg betulinic acid / g bark). SLE: 45°C; PLE: 100, 150 and 200°C. (a) Ethanol; (b) ethyl acetate solvent.

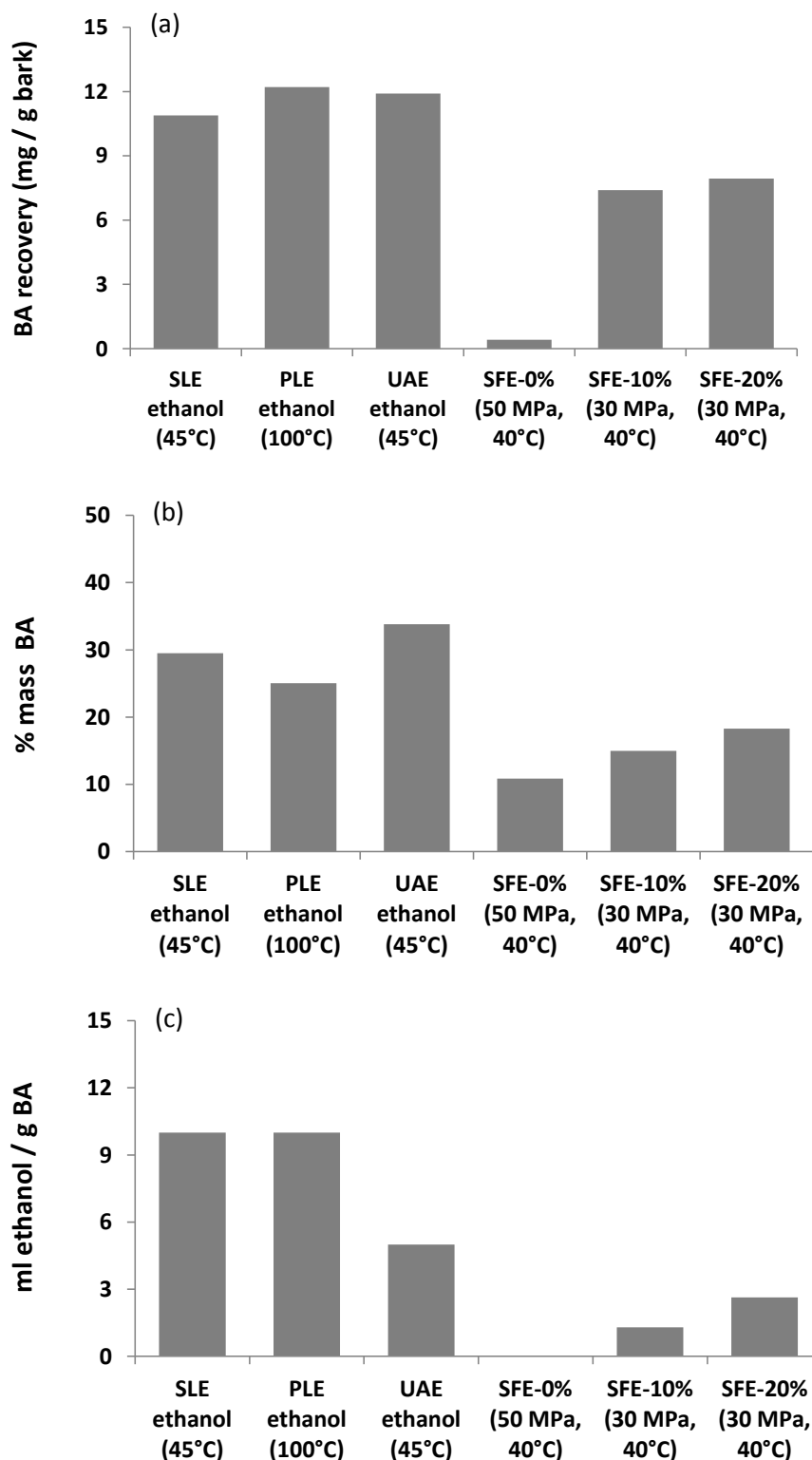


Figure 2. Comparison between the extraction methods using liquid ethanol (SLE, UAE and PLE) and SFE with 0, 10 and 20 % ethanol cosolvent. (a) BA recovery; (b) BA concentration; (c) ethanol consumption.