

Controlling concentration of bioactive components in cat's claw based products with a hybrid separation process

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Abstract

Supercritical fluid extraction (SFE) with and without entrainer combined with organic solvent extraction was used for fractionation of bioactive natural products as alkaloids and antioxidants from cat's claw (*Uncaria tomentosa* (Willd.) DC.) bark. Extracts obtained from this plant have a number of beneficial effects, including anti-inflammatory or immune stimulator. The aim of this work was to obtain extracts of enriched and limited alkaloid concentrations, good antioxidants and tannin agents. The results demonstrated that a hybrid process involving SFE is a selective method to obtain an oxindole alkaloid rich fraction (186.0 ± 3.2 mg total pentacyclic oxindole alkaloids/g extract) and a product enriched in tannins (23.8 ± 1.9 % tannin content in extract) and antioxidants ($IC_{50} = 5.88 \pm 0.54$ µg/ml extract).

Keywords

Supercritical fluid extraction; co-solvent; Cat's claw; natural alkaloid; antioxidants; tannins

Abbreviations

BHA: butylated hydroxyanisole; DPPH: 2,2-diphenyl-1-picrylhydrazyl; f: mass of CO₂ per unit of initial dry material (kg CO₂/kg dry bark); f₀: parameter to take into account soaking of bark (kg CO₂/kg initial dry bark); HAE: hydroalcoholic extract (dry basis); IC₅₀: inhibition coefficient 50 (mg/L); k: kinetic parameter (kg dry bark/kg CO₂); POA: pentacyclic oxindole alkaloids; RDM: radial diffusion method; SHE: Soxhlet extraction; TE: tannin acid equivalent; TOA: tetracyclic oxindole alkaloids; Y_{E∞}: maximum extraction yield (g extract/100 g dry bark)

1. Introduction

The market and potential of bioactive components from natural sources are continuously growing. Various extraction methods are applied to concentrate the valuable components of plants to be used in dietary and cosmetic products. Besides water or organic solvent based extractions, supercritical fluids have been used to extract target components from varied matrices, at analytical and commercial scales. [1, 2] Supercritical carbon dioxide extraction is an environmentally friendly alternative of conventional solvent extraction, as the CO₂ is regarded as safe, clean, non-toxic, non-flammable, and it is easy to remove from the extracted compounds by simply releasing the pressure. Moreover, its relatively mild critical conditions enable thermolabile compounds to be obtained without degradation. [3]

Uncaria genus is a well-known medicinal herb. *Uncaria* extracts have been employed for the discovery and utilization of their bioactive natural compounds. The plant is widely studied due to its medicinal properties, ecological and economic value. [4-9] Both *Uncaria tomentosa* (Willdenow ex Roemer and Schultes) DC. and *Uncaria guianensis* (Aublet) Gmel., from the Rubiaceae family, have been used by indigenous healers of tropical South America for the treatment of uncountable diseases and affections. They are traditionally named 'cat's claw', 'uña de gato', 'saventaro', 'garabato amarillo' or 'Katzenkralle', among others. [10] Peruvian *Uncaria tomentosa*, a woody vine, is the one with the largest number of identified active compounds. This species is found only in Central and South America [11]. In vitro and in vivo experiments, animal studies and clinical trials have revealed several beneficial properties in the extracts: cytotoxicity, anti-inflammatory, antiviral, immunostimulant, antioxidant, CNS-related response, vascular properties, hypotensive, mutagenicity or antibacterial. [12] *U. tomentosa* also is classified in one of the most important European pharmacopeias: Hager Handbuch der Pharmazeutischen Praxis [13], apart from being included in the WHO Medicinal Plants Monographs [14], British Herbal Pharmacopeia [15] and American Herbal Pharmacopeia [16].

The phytochemistry of the plant includes different groups presenting biological activity: polyphenols (tannins, procyanidins), terpenoids (sterols, glycosides or triterpenes) and alkaloids. [9]

The last ones constitute the most relevant group of bioactive components in cat's claw, as they appear in the plant in strange abundance, and are well recognized as potent medicinal compounds with herbal origin [17-19, 10]. Alkaloids can be potent drugs and poisons depending on the concentration and type, both found in tetracyclic and pentacyclic forms as well. The reported pentacyclic oxindole alkaloids (POAs) are recognized mainly as immunostimulants, while tetracyclic oxindole alkaloids (TOAs) don't present this bioactivity. [11] The total oxindole alkaloid content of different *Uncaria* extracts from different harvests was reported to be around 6 mg/g raw material, with this concentration being the highest in the bark of the plant. [8, 10, 20-21] Moreover, they are known to undergo isomerization in aqueous solutions, which is pH and temperature dependent [18, 22].

In light of this, extracts have been developed and patented, with a standardized POA (Pentacyclic Oxindole Alkaloids) content and with tested absence of TOA (Total Oxindole Alkaloids). The commercially available extracts in Western Europe, such as Krallendorn® manufactured by Immodal Pharmaka GmbH are commonly standardized so as to present a range of 1.30 – 1.75 % of oxindole alkaloid concentration, where 97 % of this concentration corresponds to pentacyclic alkaloids. The determination of isopteropodine, an alkaloid presenting the most intense immunostimulation properties, is usually involved in these standardizations. [10, 20] On the other hand, marketing cat's claw based dietary supplements is strictly restricted in some other European countries due to concerns related to its oxindole alkaloids content.

Extraction methodologies such as organic solvent extraction by maceration, decoction or Soxhlet extraction; steam distillation or liquid CO₂ extraction have been investigated on *U. tomentosa* [23]. However, the only paper reporting the use of SFE on cat's claw is that of V. Lopez-Avila and J. Benedicto [24] in which scCO₂ with and without methanol as entrainer was used for isolation of oxindole alkaloids for analytical purposes.

In this work SFE with pure carbon dioxide (scCO₂) and ethanol as entrainer is applied to the bark of *U. tomentosa*, as well as on its hydro-alcoholic extract in a two-step process. The final goal is to obtain valuable products from the drug with different applications in industry: a fraction rich in pentacyclic alkaloids, but with low levels of tannins and antioxidants, which can be used for

pharmaceutical purposes; and another fraction rich in antioxidants and tannins but with low levels of alkaloids, suitable for dietary applications.

2. Materials and methods

2.1. Materials

Bark of *Uncaria tomentosa* (two different samples), was provided by Gradiens Ltd. (Perbál, Hungary). Characterization and pretreatment of the bark is described in supplementary material. The CO₂ used for SFE of 99.5 w/w % purity was supplied by Linde Ltd. (Budapest, Hungary). All organic solvents were purchased from Molar Chemicals Ltd. (Budapest, Hungary). HPLC water and HPLC acetonitrile ($\geq 99.9\%$) were obtained from Merck Millipore Ltd. (Budapest, Hungary). Mitraphylline standard (purity $> 90\%$) was isolated and provided by Gradiens Ltd. (Perbál, Hungary). Isomitraphylline ($> 99\%$) was purchased from Phytolab GmbH (Vestenbergsgrenth, Germany). Pteropodine (Uncarine C) with purity $>97\%$ and isopteropodine (Uncarine E) with purity $> 99\%$ were purchased from Cfm Oskar Tropitzsch GmbH (Marktrechwitz, Germany).

2.2. Soxhlet extraction

2.2.1. Laboratory scale

Conventional Soxhlet extraction was performed for about 24 hours with different solvents (water, ethanol and their mixture, acetone, ethyl acetate, *n*-hexane) at ambient pressure. Soxhlet extraction was also investigated on the residue of the SFE, with absolute ethanol and acetone. The extraction was finished when the solution over the sample became colorless. The extracts were evaporated till dryness with rotating evaporator at 30 mbar pressure, and at 40 °C temperature. All extractions were carried out in triplicate.

2.2.2. Pilot scale

The grounded material was extracted without any further pretreatment. Traditional Soxhlet extraction [26] was carried out in a pilot plant apparatus, using a solution of ethanol and water in a ratio 50:50 (v/v). The drug was weighed and placed into a 5 dm³ jacketed extraction vessel, then

steeped with the solvent (H₂O-EtOH 50:50 v/v). The solvent, extraction vessel and reboiler were heated with steam. During the extraction the solvent flow rate, temperatures of the solvent vessel and the extractor were measured and registered every 30 min. Samples were taken and distilled. The process was run until the solid content of the extract became less than 0.10 %.

2.3. Supercritical Fluid Extraction

Supercritical CO₂ extractions with and without entrainer were carried out in a high pressure 5 dm³ extractor. The extractor vessel was supplied by Natex Prozesstechnologie GesmbH (Ternitz, Austria). A more detailed description of the equipment and the process can be found in previous papers [26]. When co-solvent was used, the ethanol flow was mixed with the CO₂ flow in a static mixer before entering the extractor.

Temperature, pressure in the extractor and the separator, solvent flow and density of the scCO₂ were measured and registered continuously, and samples were collected, distilled and weighed. The process was finished when the extraction yield varied less than 0.1 % between two consecutive samples.

SFE was applied on the grounded raw material as well as on the hydro-alcoholic extract, with a solvent of CO₂ with ethanol in different concentrations (0, 5, 10, 15 % w/w), a pressure ranging from 15 to 30 MPa, temperature between 35 and 55 °C and solvent to feed ratio of 10 kg CO₂·(kg dry raw material)⁻¹·h⁻¹.

2.4. Analytics

2.4.1. Oxindole alkaloids (HPLC)

An HPLC method was developed for quantitative determination of oxindole alkaloid concentration based on the method of Montoro et. al. [27]. Prior to the chemical analysis, alkaloids were isolated by partition with chloroform and Na₂CO₃ solution.

The HPLC system consisted of a degasifier, an HPLC pump (JASCO PU-1580), gradient box (JASCO LG-980-02), thermostat (Jones Chromatography Model 7955), autosampler (JASCO AS-

2057 plus), transmitter (JASCO LC-NetII/ADC) and detector (JASCO-MD-910 multiwavelength). The method was controlled and the data was evaluated using the software ChromNav.

The column used was SUPELCOSIL™ LC-18, 25 cm × 4.6 mm, filled with particles of 5 µm diameter size. An isocratic method was used, with the eluent consisting of ammonium acetate / acetonitrile 35 / 65(v/v), flow rate of 1.0 ml/min and injection volume of 10 µL. The compounds were detected at 243 nm. Calibration series for mitraphylline, isomitraphylline, pteropodine, isopteropodine were done between 0.0025 – 0.2000 mg/ml.

2.4.2. Evaluation of antioxidant activity

Antioxidant activity evaluations were performed by means of DPPH method or free radical scavenging assay [28]. A solution of the free radical 2,2-diphenyl-1-picrylhydrazyl in methanol was used. The reduction of the absorbance in solutions with different concentration of *Uncaria* samples was monitored at 517 nm. In case of extracts the samples were dissolved in methanol and the desired concentrations were then set. The residue of the bark is not soluble completely (but there are still some soluble compounds in it) so we used the same ratio solid-methanol as for extracts, then it was sonicated in an ultrasonic bath and filtered, so we obtained a final solution without solids in suspension. A spectrophotometer Camspec M501: Single Beam Scanning UV-Vis was used for the analyses. To evaluate and contrast the results, the inhibition coefficient 50 (IC₅₀) was used: the minimum concentration of the sample needed to reduce the absorbance of the samples by 50 %.

2.4.3. Evaluation of the tannin activity

Total tannin activity was determined by the radial diffusion method [29]. The assay is the precipitation of tannins in form of rings within the containing Bovine Serum Albumin (BSA) protein in agarose gel on a Petri plate. The area of the formed rings correlates with the tannin content of the sample on a straight-line basis.

3. Results and discussion

The two-step process has been investigated in two different configurations. Process parameters, plant material and solvent requirements, as well as concentration, isolation and recovery of active compounds in the extracts have been evaluated. The yields of the laboratory scale Soxhlet extractions, used as comparison, can be found in the supplementary material available online.

3.1 Solvent extraction followed by supercritical fluid extraction

In this configuration, Soxhlet extraction with a mixture of EtOH – H₂O 50:50 v/v was applied on the milled drug. The hydro-alcoholic extract (HAE) underwent further fractionation with SFE (CO₂ and ethanol as a cosolvent) as shown in Figure 1. The hydro-alcoholic extract was mixed with a carrier (cellulose) in a mass ratio 1:1, in order to turn its viscous texture into a powder forming a porous bed in the extraction vessel.

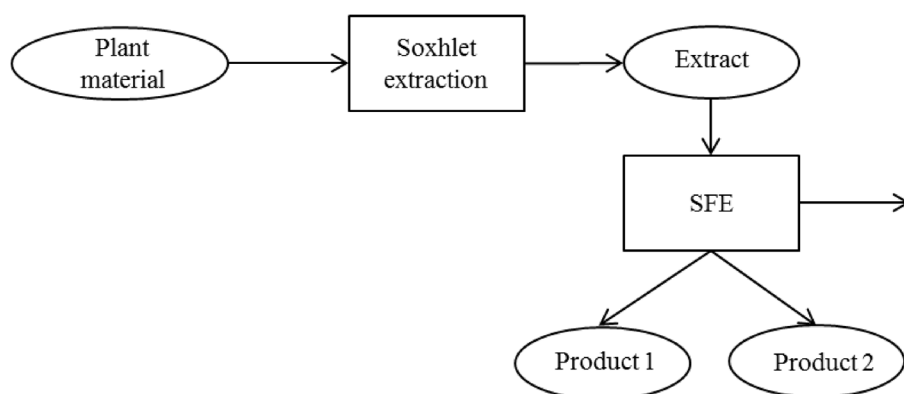


Fig. 1. Scheme of process I.: Soxhlet extraction + SFE. Product 1 is the extract, Product 2 is the residue of the SFE step.

The pilot scale hydro-alcoholic extraction yielded 15.43 g/100 g dry bark. Effect of cosolvent concentration (0-15 % EtOH) was studied in the fractionation of the hydroalcoholic extract at constant pressure of 30 MPa and 45 °C temperature. In the case of pure carbon dioxide, the pressure was varied stepwise between 10 and 45 MPa. The temperature was kept constant (45 °C) as well as the solvent-feed ratio, with a solvent flow of 5 kg/h and a feed of 100 g.

The experimental results on total extraction yield of the hydro-alcoholic extract with scCO₂ and different concentration of cosolvent are shown in Table 1.

Table 1.

Influence of cosolvent concentration in the SFE of Uncaria hydroalcoholic extract.

P (MPa)	Solvent	T (°C)	Y _E (g/100 g HAE)	Y _E (% g extract / g dry bark)
30	CO ₂	45	1.05	0.16
30	CO ₂ + 5 % EtOH	35	2.30	0.35
30	CO ₂ + 10 % EtOH	45	5.82	0.89
30	CO ₂ + 15 % EtOH	45	7.96	1.23

Extraction with scCO₂ led to a yield of barely 1 % of the HAE. Addition of ethanol as a cosolvent increased the extraction yield due to the increased polarity of the supercritical fluid. The extraction yield increases with the concentration of cosolvent up to 8 % with 15 % ethanol.

Figure 2. shows the antioxidant activity measured with DPPH method (in terms of IC_{50}), along with tannin activity of selected samples of the process I. The column in Figure 2. corresponds to the antioxidant activity of a synthetic antioxidant (BHA: butylated hydroxyanisole). The following columns correspond to Soxhlet extracts with different solvents of the bark, and finally residues from SFE on the hydro-alcoholic extract (HAE) of cat's claw bark. None of the SFE extracts, regardless of the co-solvent concentration used, showed reasonable antioxidant activity.

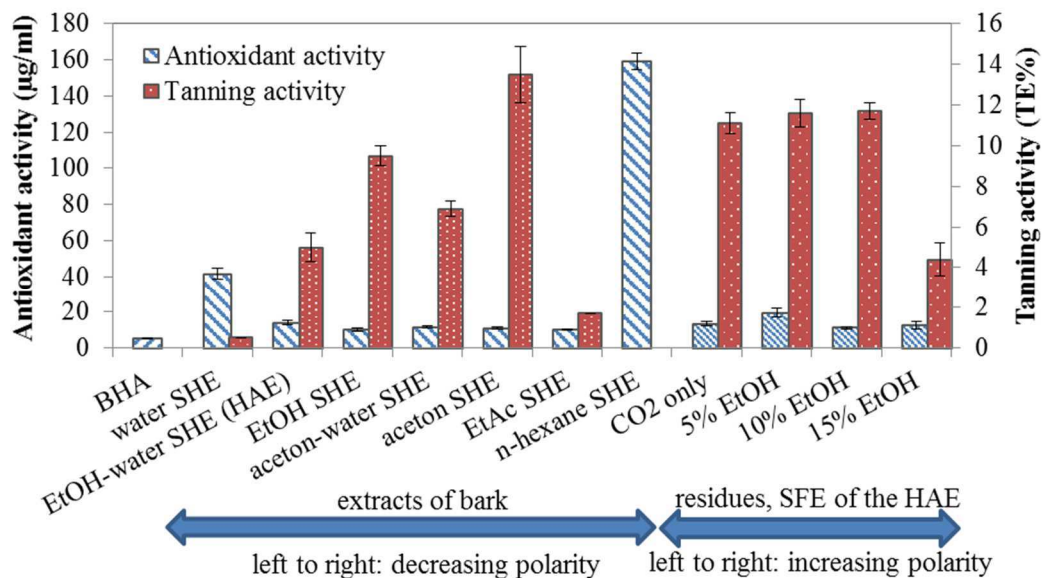


Fig 2. Antioxidant activity and tanning activity of different products from process I. SFE: 30 MPa, 45 °C, cosolvent concentration presented in the figure.

The extracts obtained with semipolar solvents (acetone, ethanol, acetone-water) are the ones having good antioxidant activity and tanning agents content. Extracts with EtOH-H₂O mixtures, acetone, EtOH, acetone mixtures and ethyl acetate show results for IC_{50} comparable to the synthetic antioxidant available (BHA). Moreover, residues from SFE of the hydro-alcoholic extract also present higher antioxidant and tanning activity than the HAE. That means that with SFE we are able to fractionate the antioxidants and non-antioxidant components of *Uncaria*, and also tanning agent rich and poor samples. There is no clear relationship between tanning and antioxidant activities,

however the two properties can be correlated, suggesting not only the tanning components are responsible for the free radical scavenging activity of the cat's claw.

Figure 3. shows the total pentacyclic oxindole alkaloid (POA) concentration in extracts obtained from SFE on the hydroalcoholic extract. The last column shows the results of SFE of the pure drug at medium concentrations of cosolvent and same pressure and temperature (30 MPa, 10 % EtOH).

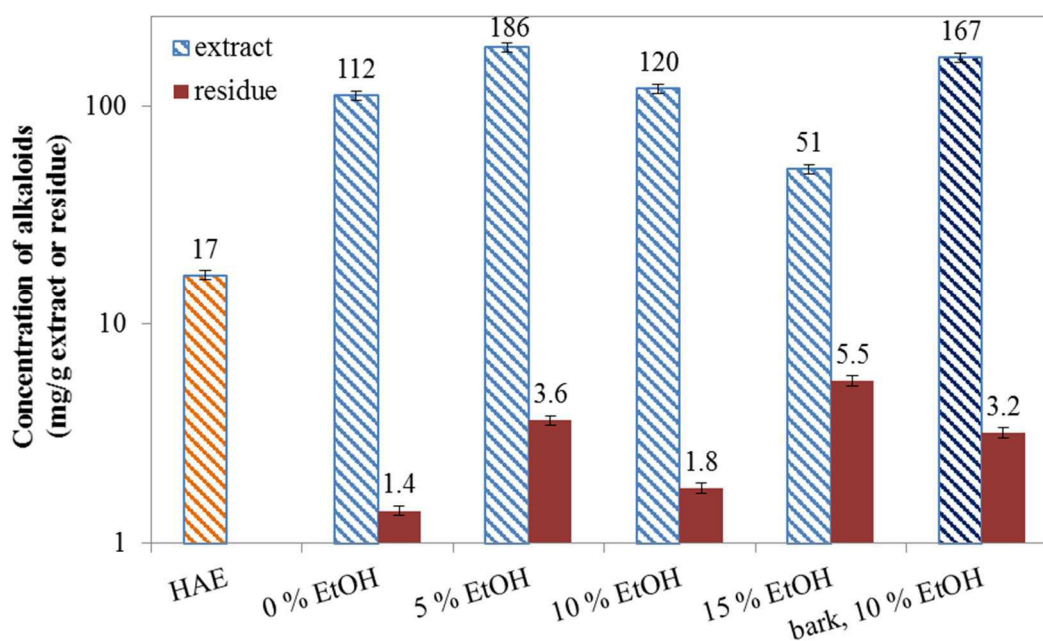


Fig 3. Total oxindole alkaloid concentration (logarithmic scale) in the HAE (hydroalcoholic extract), SFE extracts and residues of HAE and of the bark. All SFE were performed at 30 MPa and 45 °C with varied ethanol concentration as given in the figure. Error bars show the analytical uncertainty only.

The alkaloid contents of the extracts obtained by the first configuration (Soxhlet extraction followed by SFE) varies noticeably with the process parameters. The lower alkaloid concentration in the alcoholic extract is due to the high recovery of other components. Using further SFE for fractionation of the alcoholic extract appears to be a selective method for oxindole alkaloids isolation. Using SFE of the hydro-alcoholic extract with pure CO₂, the concentration of alkaloids highly increases with the pressure, from 10 to 30 MPa (fraction shown in the graph). By adding ethanol as entrainer, this concentration increases up to 10 % EtOH. The optimum appears at 30 MPa and supercritical CO₂

with 5 % EtOH. This concentration (186 mg/g extract) is around ten times higher than the initial hydro-alcoholic extract, and is high enough to envisage a cat's claw based product enriched in pentacyclic oxindole alkaloids. On the other hand, by the analysis of individual alkaloids, it was observed that isopteropodine appears in remarkably high concentration in fractions obtained at 30 MPa, without EtOH and with 5 %. This alkaloid is reported to have the most potent immunostimulation properties [20] and is determined for standardization of *Uncaria* extracts in Western Europe.

The recovery of oxindole alkaloids in the samples obtained after fractionation of the hydroalcoholic extract of cat's claw bark (configuration 1) is relatively low due to the fact that the recovery of these compounds with EtOH – H₂O is not complete. The maximum recovery is obtained in the extract from fractionation of the hydro-alcoholic extract with 10 % EtOH, with 1.06 mg POA/g dry plant material.

3.2 Supercritical fluid extraction and solvent extraction on the residue

In this configuration, supercritical fluid extraction with 10 % of cosolvent is applied on the milled drug, and the residue obtained is extracted with Soxhlet using ethanol or acetone, as shown in figure below (Figure 4):

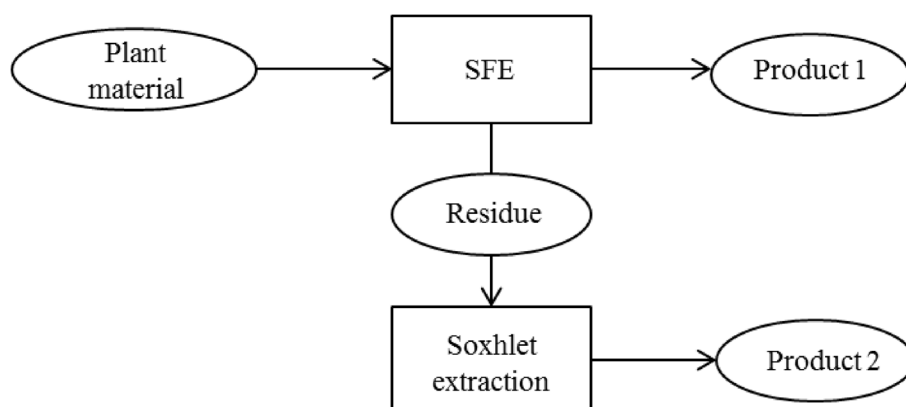


Fig.4. Scheme of process II.: SFE followed by hydro-alcoholic Soxhlet extraction of the residue. Product 1 is the extract of SFE, Product 2 is the extract of the HAE.

The influence of pressure and temperature were investigated on the milled bark of *Uncaria tomentosa*. Consecutive extractions were performed, with a fixed solvent of CO₂ and 10 % ethanol,

and a fixed solvent flow rate of 5 kg/h and solvent to mass ratio of 10 kg CO₂ · h⁻¹/kg of raw material. The repeatability was tested by doing repetitions of the extraction at the same conditions, at 22.5 MPa and 45 °C, and 30 MPa and 45 °C.

The SFE curves were represented and fitted using a modified form of Brunner's equation [30] taking the mass flow of CO₂ consumed per unit of plant material as the independent variable:

$$Y_E = Y_{E\infty} \cdot [1 - \exp(-k \cdot (f + f_0))] \quad (1)$$

where:

Y_E = extraction yield (g extract/100 g dry bark)

$Y_{E\infty}$ = maximum extraction yield (g extract/100 g dry bark)

f = mass of CO₂ per unit of initial dry material (kg CO₂/kg dry bark)

f_0 = parameter to take into account soaking of bark (kg CO₂/kg initial dry bark)

k = kinetic parameters (kg dry bark/kg CO₂)

Yield curves are plotted in Figure 5. The pressure and temperature have no significant effect on the extraction curves and yields. Statistical evaluation methods are detailed in supplementary material.

$Y_{E\infty}$ is 1.44 ± 0.07 %, value of k is 0.037 ± 0.002 kg initial dry material/ kg CO₂.

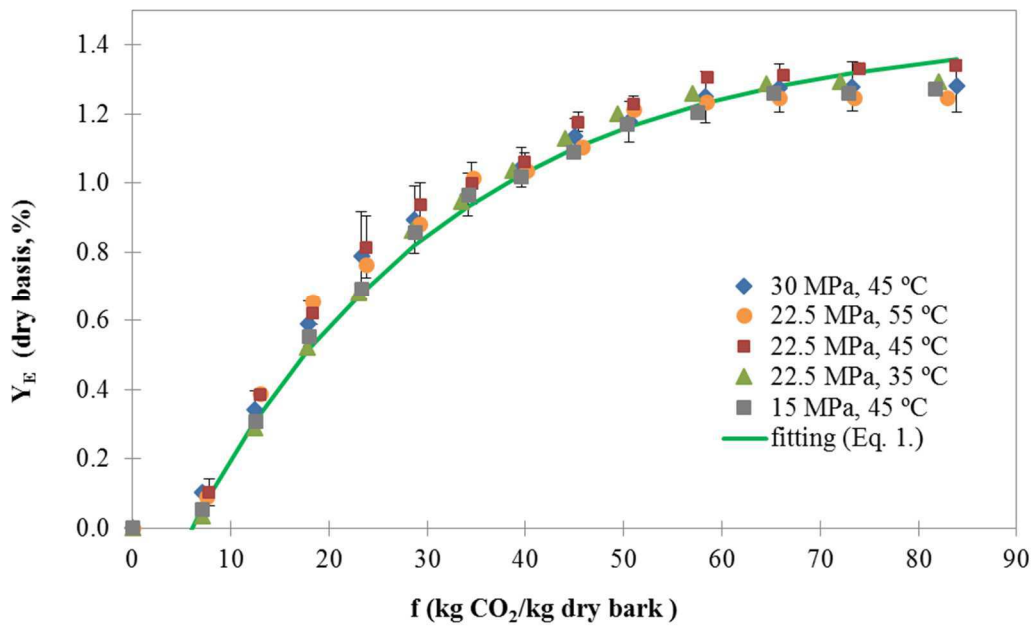


Fig. 5. Influence of the pressure and temperature in the SFE of cat's claw bark (scCO₂ + 10 % EtOH). Experimental data and fitting of Eq. 1.

Figure 6. shows the antioxidant activities (IC₅₀, the smaller the better) and the tanning activities of process II samples.

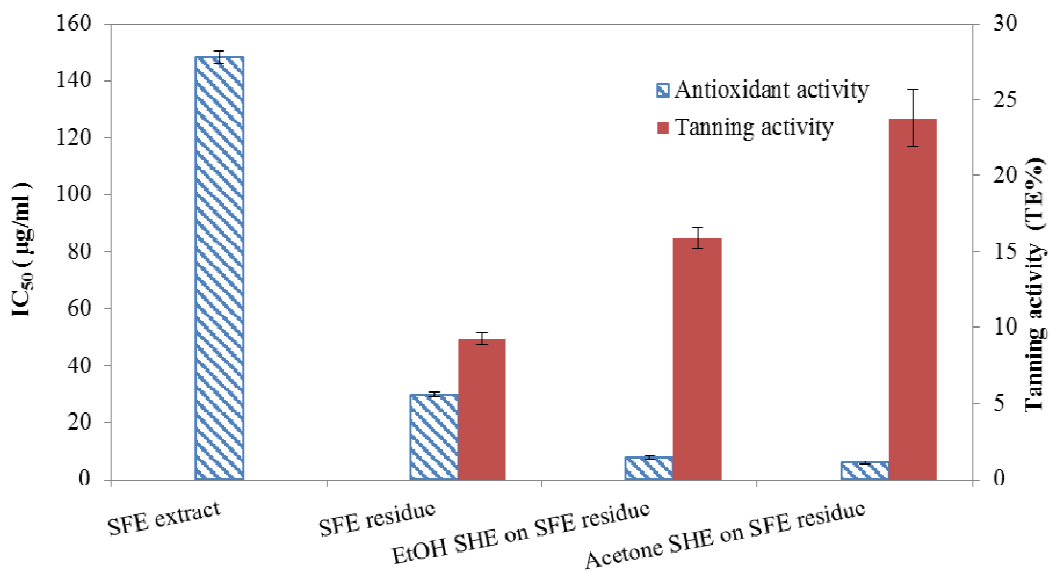


Fig 6. Antioxidant activity and tanning activity of various products from process II.

The extract 1 (SFE (CO₂ + 10 % EtOH) of the bark) did not show any relevant antioxidant activity and has negligible tanning activity. However, the residue, shows medium antioxidant activity (in terms of IC₅₀ of the residue) and tanning activity (in terms of % g tannic acid equivalent/g residue), and these values improve noticeably when the residue is fractionated with organic solvents (acetone and EtOH).

Concentration of total pentacyclic oxindole alkaloids, quantified by the developed HPLC method, is presented in Fig. 8.

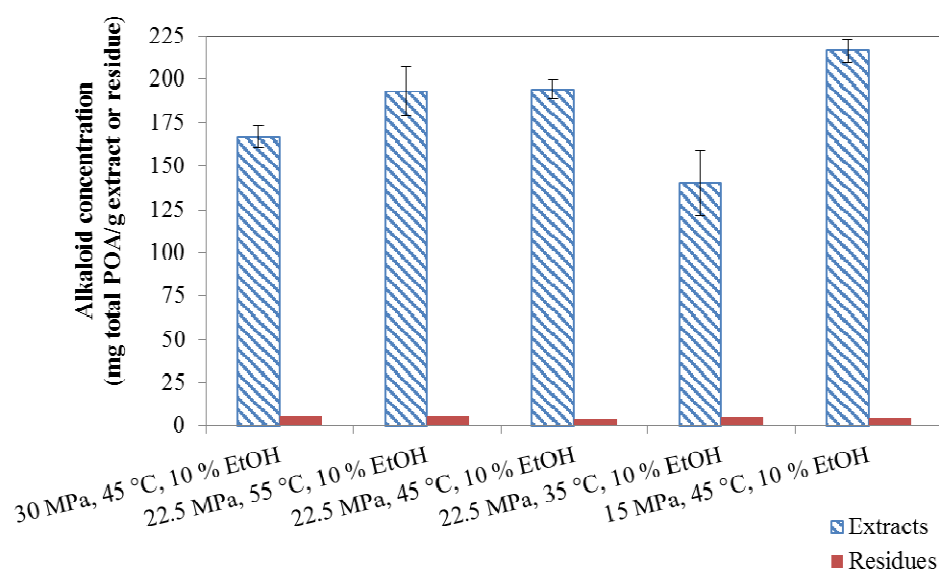


Fig.8. POAs concentration in supercritical extracts and residues from cat's claw bark.

The temperature does not seem to have a positive influence on the extraction of these compounds at temperatures higher than 45 °C, therefore this value seems appropriate for the optimization of energetic and economic conditions. On the other hand, an increase in pressure causes a slight decrease in the concentration of POAs in the range studied (15 – 30 MPa). In order to select an optimum pressure for this process, the lowest pressure seems the best choice in financial and energetic terms, but the extraction of other compounds, operation of the process, etc. may be taken into account as well.

The recovery is still noticeably higher in the residues than in the extracts due to the low extraction yields. The total recovery adding up extracts and residues in every extraction (6 – 8 mg POAs/g dry

plant material) is consistent with the literature. Pilarski et al. [32] reported the presence of 725.34 mg total main alkaloids in 100 g bark of *Uncaria* (7.2534 mg/g). The results calculated are consistent in the different extraction conditions.

Comparing the results of alkaloids, tannins and antioxidant activity, extracts with good antioxidant activity present medium concentration of alkaloids. The supercritical extract with 10 % EtOH, which is the sample with higher concentration of alkaloids, is non-antioxidant or pro-oxidant. The supercritical residues extracted with acetone or EtOH show slightly less concentration of alkaloids than the acetone and ethanol extracts on the pure *Uncaria*, but they are better antioxidants. These data suggest that there are more components responsible for the antioxidant activity, as reported in the literature [32].

On the other hand, fractions with low concentration of alkaloids show good antioxidant and tannin activity, supporting previous studies that reported that some *Uncaria* species contain tannins with antioxidant activity, which are also responsible for some pharmacological effects [33, 34].

3. Conclusions

Two-step extraction processes were developed for extraction and concentration of bioactive components of *Uncaria tomentosa* bark.

The bark of the plant is milled and extracted with conventional Soxhlet extraction with a mixture of ethanol and water 50:50 v/v. Secondly, the dry alcoholic extract undergoes further supercritical fluid extraction with CO₂ and ethanol cosolvent. In the second scheme, the milled bark is extracted by SFE with scCO₂ and 10 % EtOH, at constant pressure, temperature and solvent flow to feed ratio. The residue from this first extraction is extracted by Soxhlet with organic solvents, namely ethanol and acetone.

HPLC results showed a high selectivity for the alkaloids in extracts obtained with scCO₂ modified with ethanol. Standardized extracts from *Uncaria* used in Western Europe are meant to contain 1.30 – 1.75 % oxindole alkaloids with approximately 97 % of the total alkaloid content accounting for pentacyclic alkaloids [10]. The concentration of these compounds in the products obtained in this

study, at optimum conditions is high enough to envisage a product based on isolation of the alkaloid fraction, which can be applied in medicine industry.

The second configuration (SFE followed by organic solvent extraction on the residue) leads to a product with the highest concentration and recovery of alkaloids, as well as a second product with very high antioxidant activity and tannin content. However, in this configuration high quantities of plant material are used in the high pressure extraction step, while in the first configuration the volume treated with SFE is reduced drastically, but on the other hand the hydro-alcoholic extract should be mixed with a carrier before the SFE.

Any of the two proposed process schemes is relatively easy to implement at commercial scale, and can be used to obtain products with high content of alkaloids, applicable for pharmaceutical industry, and others rich in antioxidants and tannins but with low levels of alkaloids, that might be used in food or for cosmetic purposes.

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Supplementary material

Characterization of Uncaria tomentosa bark

The moisture content of the plant material was determined by the standard method described in the European Pharmacopeia. In the same way, oil content was evaluated by standard method (hexane Soxhlet extraction). Characteristics of the drug samples are given in Table 1.

Table 1. Moisture and oil content and density of the two studied samples of cat's claw bark.

Sample	Moisture content (%)	Oil content (%)
<i>Uncaria A</i>	4.53 ± 0.43	1.27 ± 0.23
<i>Uncaria B</i>	7.00 ± 0.16	0.34 ± 0.02

The cat's claw bark was milled in a mechanical cutting mill up to 1 mm sieve size. The particle size distribution of the starting material was determined by using sieves of various mesh sizes and calculating the fraction weights. The characteristic particle size (or statistical average diameter, (that above which 36.8 % of particles would lie)) of every material was calculated with the statistic method by Rossin-Ramler-Sperling-Bennet (RRSB) [1] using the software STATISTICA 12. The statistical average diameter obtained with RRSB is shown in the table below, as well as the degree of uniformity around this value (n).

Table 2. RRSB model parameters for the two studied samples of cat's claw bark.

Sample	Statistical average diameter (mm)	Degree of uniformity
<i>Uncaria A</i>	0.1868 ± 0.0297	1.4101 ± 0.0611
<i>Uncaria B</i>	0.2680 ± 0.0611	1.6420 ± 6.6600

Laboratory scale Soxhlet extraction yields are presented in Table 3 with various solvents.

Table 3. Soxhlet extraction yields of *Uncaria B* samples.

Solvent	Yield (%)
n-hexane	0.34 ± 0.02
ethyl acetate	4.48 ± 0.68
ethanol	19.71 ± 0.63
acetone	13.39 ± 0.53
acetone-water 50:50	15.78 ± 1.08
ethanol-water 50:50	24.84 ± 1.38
water	20.22 ± 1.62

Statistical evaluation of the extraction curves

Statistical analysis [2] was performed to decide whether pressure or temperature do have significant effect on the parameters of the regression curve fitted on the modified Brunner's equation.

The null hypothesis to be tested is that at different conditions the SFE curves do not differ more from each other than the ones fitted on the data from the repeated experiments run with the same conditions. This means that the effects of the pressure and the temperature do not cause additional variation to the variation of the run-to-run replication.

The applied statistical method is the general linear test. The F -test has the following form:

$$F = \frac{(SSC - SSD)/(v_C - v_D)}{SSR/v_R}$$

where SSC is the residual sum of squares with v_C degrees of freedom for the model fitting one common curve for the data from the different conditions, SSD is the residual sum of squares with v_D degrees of freedom for the model fitting separate curves for (the data from) the different conditions and SSR is the residual sum of squares with v_R degrees of freedom for the model fitting one curve for the data sets of the same conditions.

In this study SSC and SSD is calculated from the three different conditions, where no repetition is made (run numbers 2, 4, and 5) and SSR is calculated from the two-two data sets where the repeatability is tested (run numbers 1 and 3).

The result is summarised in Table 1:

sum of squares		degrees of freedom	
SSC	0.03185	v_C	36
SSD	0.01586	v_D	30
SSR	0.10245	v_R	46

The F -test statistic is 1.197 and the p -value is 0.6751. This implies that the null hypothesis of no additional variance cannot be rejected, thus the differences in the experimental conditions (temperature, pressure) do not cause additional variability in the experimental results.

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