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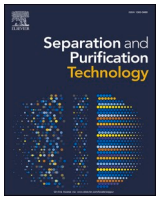

Sequential extraction of oxindole alkaloids from *Uncaria tomentosa* leaves by green pressurized solvents

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ABSTRACT

Uncaria tomentosa (Cat's Claw) is a woody climbing vine known for its therapeutic effects, which have been validated in clinical trials. The leaves, often discarded in traditional uses, contain high quantities and a wide variety of oxindole alkaloids, such as rhynchophylline (Rhy), isomytraphylline (Imyt), or mitraphylline (Myt), with Myt typically being the major alkaloid found in extracts of this species. In this study, sequential extraction in two steps was employed to recover oxindole alkaloids. In the first step, pressurized liquid acetone was used, extracting approximately 17 % of the raw material mass, with no detectable alkaloids in the extract. In the second step, CO_2-expanded liquid ethanol (25 % w/w) was used under varying temperature, pressure, and solvent density conditions. Extracts enriched with the oxindole alkaloids Rhy, Imyt, and Myt were obtained from the 2nd step extraction, with ratios ranging from 39:61 to 62:38 (Rhy:Myt + Imyt). These extracts demonstrated significant inhibition of lipoxygenase (up to 95 %) and acetylcholinesterase, indicating potential anti-inflammatory and neurological applications. Moreover, these effects were positively correlated with the proportion of Rhy relative to Myt + Imyt. Based on the alkaloid content and the observed potential bioactivities, optimized parameters, robustness and process stability were assessed using deterministic and stochastic statistical methods to maximise Rhy and minimise Imyt and Myt. Finally, this study presents the development and optimization of a robust process to obtain extracts with anti-inflammatory and neurological potential, adding value to *U. tomentosa* leaves and promoting the sustainable use of this plant as a raw material.

1. Introduction

Uncaria tomentosa (one of the species known as Cat's Claw) is a high-climbing woody vine renowned worldwide for its therapeutic applications. The main product derived from this plant is the stem bark, which is sold in its dried form for use in infusions and capsules [1]. The growing interest in the production of phytotherapeutics from this plant has raised concerns about managing its residues, typically comprising unused plant parts that may contain valuable bioactive compounds. Notably, the stem bark, which is the most extensively studied part of the plant, contains approximately 6 mg of oxindole alkaloids per gram [2]. Since the stem bark is the most used part, the leaves often become waste. However, studies indicate that the leaves may contain up to seven times more alkaloids than the stem bark [3].

The primary alkaloids found in *U. tomentosa* include pentacyclic and tetracyclic oxindole alkaloids such as rhynchophylline (Rhy), isorhynchophylline, mytraphylline (Myt), isomytraphylline (Imyt), pteropodine, isopteropodine, speciophylline, and uncarine F [4]. Extracts enriched with these alkaloids have demonstrated therapeutic potential against numerous diseases, including cancer and Alzheimer's disease [5–10]. A Phase II clinical trial revealed that a *U. tomentosa* extract containing 5 % Myt improved the quality of life and reduced fatigue in terminally ill cancer patients with nineteen different cancer types [11]. Additionally, *U. tomentosa* extracts have shown anti-inflammatory and anti-proliferative activities, which are particularly significant given the close relationship between chronic inflammation and cancer [12]. Aqueous extracts of *U. tomentosa* bark have exhibited acetylcholinesterase (AChE) inhibition, likely due to oxindole alkaloids, highlighting their potential in treating Alzheimer's disease [13]. Furthermore, Rhy has been shown to cross the blood–brain barrier and has demonstrated therapeutic potential against neurological disorders such as Alzheimer's disease [14–21]. Despite the importance of Rhy,

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previous studies have established Myt as the major alkaloid in the leaves, roots, and stem bark [4,22,23]. Given this, it is essential to develop extraction methods that yield extracts with a varied composition of these alkaloids, thereby enabling the evaluation of potential synergistic effects.

To obtain extracts enriched with these alkaloids, conventional extraction methods often use solvents such as methanol, ethanol, and water [6,8–10]. Other solvents, including ammonium hydroxide, dichloromethane, and hydrochloric acid, have also been employed to enrich Myt content for subsequent isolation [7,24]. Supercritical carbon dioxide ($scCO_2$) modified with methanol or ethanol has proven to be effective in solubilizing Myt and Rhy [2,25,26]. However, the isomerization of these alkaloids is influenced by extraction parameters such as temperature, pH, solvent polarity, and the type of extraction process. For instance, Kaiser et al. [27] found that reflux extraction, turbo-extraction, and static maceration caused significant isomerization of oxindole alkaloids, while ultrasound-assisted extraction and dynamic maceration minimized this effect.

The use of pressurized fluids for extracting oxindole alkaloids from *U. tomentosa* has been explored by a few researchers [2,25,26]. López-Avila et al. [25] conducted sequential extractions from plant roots using pure $scCO_2$ in the first step, followed by $scCO_2$ modified with methanol in the second step. They observed two Myt isomers in the first step and five in the second step, with Rhy being only present in the latter. Calvo et al. [2] employed $scCO_2$ modified with ethanol to extract alkaloids from stem bark, varying the ethanol content from 0 to 10 %. Additionally, Fu et al. [26] developed a sequential extraction method and a chromatographic separation for alkaloids in the same genus, using $scCO_2$ modified with ethanol, adsorbents, and diethylamine.

The addition of a co-solvent to $scCO_2$ alters its solubility, enabling the extraction of compounds that are otherwise inaccessible with pure $scCO_2$. Alternatively, CO_2 can act as a co-solvent in gas-expanded liquid extraction, modifying the solvent's polarity and solvation power through changes in viscosity, solute diffusivity, and interfacial tension [28]. Despite its lipophilic nature, CO_2 can act as a Lewis acid or base [29]. The polarity of the solvent is directly linked to the isomerization of tetracyclic and pentacyclic oxindole alkaloids. Polar solvents lower the activation energy of the zwitterionic isomerization intermediate, while nonpolar solvents stabilize lipophilic isomers at equilibrium [30,31]. Incorporating CO_2 as a co-solvent in polar solvents may mitigate or inhibit isomerization during extraction.

In addition, ecological and sustainability aspects must be considered in the selection of raw materials, as well as in the choice of components involved in the extraction process, particularly solvents. Regarding the raw material, the use of leaves generally presents challenges, such as the need for a depigmentation step and/or removal of other compounds present in the extract that may compromise its quality, either by affecting the alkaloid content or the potential biological activities of the obtained extracts [32,33]. These requirements may need additional purification steps, leading to an unfavourable cost-benefit evaluation of the developed process [34].

Such limitations may be related to the fact that the leaves of *U. tomentosa* are significantly less explored compared to the stem bark and are often treated as waste during stem bark collection. With the growing use of *U. tomentosa* extracts, including in clinical trials, demand for these extracts is expected to increase, and innovative solutions must be developed to enable the use of leaves in the production of alkaloid-enriched extracts.

Another crucial aspect in the extraction of bioactive compounds is the choice of solvent. Beyond ecological and environmental concerns, extracts should preferably be obtained using green extraction methods, which reduce or eliminate the risk of patient exposure to toxic solvent residues. In this context, pressurized green solvents exhibit desirable properties, as they can mitigate risks related to toxicity and biodegradability, while also offering process advantages such as lower solvent consumption, reduced extraction time, and the potential for solvent recycling [35–37].

Based on these findings, this study aimed to investigate the sequential pressurized green fluid extraction of oxindole alkaloids from *U. tomentosa* leaves. The primary objective was to enhance the concentration of oxindole alkaloids in the plant matrix by maximizing the removal of non-oxindole alkaloids in the first step, followed by selective recovery of oxindole alkaloids in the second step. Process parameters such as temperature, pressure, solvent density, and extraction kinetics were evaluated. Given the common predominance of Myt among the alkaloids in *U. tomentosa* extracts and the potential for isomerization during extraction, correlations between Myt and Imyt contents were analysed concerning process parameters. Additionally, Rhy contents were quantified due to their noted biological properties.

Although Myt is frequently reported in the literature as the major oxindole alkaloid in *U. tomentosa* extracts, the key innovation of this study lies in the strategic design of the extraction process, which, for the first time, employed CO_2-expanded ethanol to obtain extracts enriched in Rhy, with varying proportions of Rhy relative to Myt and Imyt. To support this approach, advanced data analysis techniques, both deterministic and stochastic, were applied to explore and model the experimental data, establishing a mathematical framework that describes the composition and relative proportions of these alkaloids as a function of the independent process parameters studied. Additionally, the biological potential of the extracts was assessed through *in vitro* inhibition assays of lipoxygenase and AChE, highlighting their potential anti-inflammatory and anti-Alzheimer activities. These findings informed the optimization and capability studies of the extraction process, supported by both descriptive and Bayesian statistical approaches.

2. Materials and methods

2.1. Chemicals and raw material

Carbon dioxide (99.998 %, CAS number 124–38-9) was obtained from Praxair (Portugal). Acetone (99.5 %, CAS number 67–64-1), ethanol (≥99.8 %, p.a., CAS number 64–17-5), ammonium acetate (HPLC LiChropur®, CAS number 631–61-8), linoleic acid (99 %, CAS number 60–33-3), soybean lipoxygenase (Type V, ammonium sulfate suspension, 500,000 – 1,000,000 units/mg protein, CAS number 9029–60-1), acetylcholinesterase (AChE, Type VI-S, 500 U/mg protein, CAS number 9000–81-1), 3-carboxy-4-nitrophenyl disulfide (DTNB, ≥98 %, CAS number 69–78-3), acetylthiocholine iodide (AChI, ≥98 %, CAS number 1866–15-5), and tris(hydroxymethyl)aminomethane (Tris buffer, CAS number 77–86-1) were purchased from Sigma-Aldrich (Portugal). Rhy (99 %, CAS number 76–66-4) was purchased from ChemCruz (USA), while Imyt (99 %, CAS number 4963–01-3) and Myt (99 %, CAS number 509–80-8) were obtained from ChromaDex (USA). Hydrochloric acid (HCl, 37 %, p.a., CAS number 7647–01-0) was purchased from Carlo Erba (France).

The leaves of *U. tomentosa* were donated by the Brazilian Agricultural Research Corporation (EMBRAPA, Belém, PA, Brazil). The leaves were dried at approximately 37 °C in a forced air circulation oven and then comminuted using a knife mill (KSM 2, Braun) to obtain a particle size fraction between 48 and 24 mesh, with a moisture content of 6.5 ± 0.1 % (w/w). The processed sample was stored in polyethylene bags for subsequent analysis.

Moisture content quantification of the raw material was conducted using thermogravimetric analysis. A comminuted sample (6.4 ± 0.1 mg) was analyzed using a differential scanning calorimetry/thermogravimetric analysis instrument (DSC/TGA, SDT Q600, TA Instruments, Canada). The analysis was performed with a temperature ramp of 10 K/min from 298 K to 473 K under a nitrogen gas flow of 100 mL/min. The moisture content was determined by measuring the mass loss of the sample at 373 K until a constant weight was achieved. All experiments were conducted in duplicate.

2.2. Pressurized fluid extraction

Since the primary objective of this study was to obtain extracts enriched in Rhy, Myt, and Imyt, the extraction process was designed in two sequential steps. The first step aimed to increase the concentration of oxindole alkaloids in the raw material and in the extract obtained during the second step by selectively removing compounds that would, at least partially, be extracted in the second step.

High-pressure fluid extractions were conducted using an apparatus previously described [38], equipped with a 2×10^{-7} m^3 extraction column and using approximately 6 g of comminuted leaves per sequential extraction. For both steps, the solvent flow rate was evaluated at three levels, selected to optimize the extract yield. The extraction time for the first step was determined by visually assessing the extract and selecting the duration that minimized greenish pigmentation. In contrast, the extraction time for the second step was determined by maximizing the extracted mass.

Based on prior studies on high-pressure fluid extraction and oxindole alkaloid isomerization, as well as preliminary tests, suitable solvents were selected. Pressurized liquid acetone was used as the solvent for the first step, where the main objective was to remove non-oxindole alkaloids from the raw material and prevent alkaloid isomerization in the subsequent step. Acetone extraction was carried out at 328 K and 15 MPa, with a static extraction period of 5 min and a dynamic extraction period of 6 h, at a flow rate of 2.28×10^{-3} kg/min.

For the second step, CO_2-expanded liquid ethanol (CO_2-XLE, 25 % w/w) was used as the solvent. This step was conducted at 313 and 333 K and pressures of 16, 27, and 38 MPa, with a static extraction period of 1 h and a dynamic extraction period of 8 h, at a total flow rate of $\sim 3.36 \times 10^{-3}$ kg/min. A wet gas meter (Dwyer Instruments, GFM 2109) was used to measure the CO_2 flow rates. The pressurized liquid flow rates were calculated based on the estimated densities of CO_2-XLE (25 % w/w) and acetone [39,40], as well as on the pump output flow rate.

The second extraction step was performed using a full factorial design, where two temperature levels (313 and 333 K) were combined with three pressure levels (16, 27, and 38 MPa). The two steps were conducted sequentially without removing the sample from the extraction vessel. Residual liquid in the system from the acetone step was purged before the second step. All experiments were conducted in duplicate.

For preliminary tests and comparative purposes, a conventional subsequent ethanol extraction was performed after the acetone step. The raw material-to-solvent ratio (w/v) was 1:20, and the extraction was carried out at 351 K and atmospheric pressure (0.1 MPa). The extraction involved direct contact of the raw material with the solvent for 9 h, followed by solvent evaporation under reduced pressure using a rotary evaporator (Büchi® Rotavapor® R-210, Germany). Extract yields were calculated on a dry basis (d.b.) and stored in glass flasks at 255 K under a nitrogen atmosphere and in the absence of light.

2.3. Analytical methods: identification and quantification

All extracts were analyzed using thin-layer chromatography (TLC) with silica gel plates (20 cm × 20 cm, 0.2 mm thickness, Merck) as the stationary phase. The mobile phases used for elution were ethyl acetate-formic acid-glacial acetic acid–water (100:11:11:27, v/v) for phenolic compounds [41], and ethyl acetate-n-hexane (95:5, v/v) for oxindole alkaloids detection [42]. Mayer's reagent and NP-PEG solution were applied for the visualization of alkaloids and phenolic compounds, respectively [41]. Extracts were diluted in ethanol at a concentration of 5 mg/mL, with 10 μL of extracts obtained using CO_2-expanded liquid ethanol and 200 μL for other extracts applied to the silica gel plates.

The extracts were further analyzed by Ultra-Fast Liquid Chromatography coupled with a diode array detector (UFLC-DAD) for the identification and quantification of Rhy, Myt, and Imyt. The chromatographic analysis was performed using an RP-C18 column (5 μm, 250 × 4 mm, Sigma-Aldrich, Portugal). The UFLC-DAD system was equipped with a Shimadzu C-20AD pump and a diode array detector (Shimadzu, Model SPD-M20A IVDD). The mobile phase consisted of 30 mM ammonium acetate (A) and a methanol:acetonitrile mixture (1:1, B). The gradient elution program started at 60:40 (A:B, v/v) at 0 min and progressed to 30:70 (A:B) over 30 min, at a flow rate of 1 mL/min. The diode array detector was set to 245 nm, and the injection volume was 5 μL [4]. Calibration curves for each alkaloid were prepared using analytical standards, with linear fitting and coefficients of determination (R^2) $\geq$ 0.99. All analyses were performed in duplicate to ensure reproducibility.

2.4. Data analysis: univariate and multivariate analysis, modelling, optimization, robustness and process performance

Data analysis was conducted using JMP® Pro 17.0.0 (SAS Institute Inc., USA) and the GPyOpt 1.2.6 library (University of Sheffield, UK) in Python™ 3.12 64-bit (Python Software Foundation, USA). Independent parameters (X variables) included pressure (P), temperature (T), solvent density (ρ), and extraction time (t). Dependent parameters (Y variables) were the extract yield and the contents of Rhy, Myt, and Imyt in the extracts.

Pairwise correlations (r) were assessed for the studied parameters. Principal Component Analysis (PCA) was used to examine the qualitative effects of X variables on the combined Y variables. The Y variables were modelled as functions of the X variables, identified as relevant in the pairwise correlations, assuming consistent effects of the X variables across all Y variables. Model performance was evaluated using R^2, root mean square error (RSME), lack of fit and significance (*p*-value) tests.

For model validation, 25 % of the data (60 values for each Y variable) were randomly excluded from the fitting process and used solely for validation. Optimization studies employed desirability functions constructed in JMP, along with stochastic methods using Bayesian optimization from the GPyOpt library.

Robustness was assessed based on the derivative of the models to noise parameters P or ρ, assuming T and t were well-controlled. Robust combinations of X variables were identified when partial derivatives of the models approached zero.

Process performance for optimized and robust X combinations was evaluated by simulating 10,000 Y values using the Monte Carlo method (JMP). Stability indices, Upper Control Limits (UCL), and Lower Control Limits (LCL) were calculated for these simulations. Control limits were estimated using the moving range chart method, and RMSE values for each Y variable were used as standard deviations for random effects in the simulations.

2.5. Lipoxygenase and acetyl cholinesterase inhibition assays

The soybean lipoxygenase inhibition assay was performed as described by Sircar et al. [43] with modifications to evaluate the indirect anti-inflammatory activity of the obtained extracts. This method measures the inhibition of 13-hydroperoxyoctadeca-9,11-dienoate formation from linoleic acid at 234 nm and 298 K. The Michaelis-Menten kinetic model was used to determine lipoxygenase concentration.

For each assay, 2 mL of linoleic acid (100 μM, in 0.1 M potassium borate buffer, pH 9) were mixed with 10, 20, or 30 μL of extract solution, control, or reference drugs (0.5 mg/mL in ethanol), and 50 μL of soybean lipoxygenase solution (1 mg/mL in 0.1 M potassium borate buffer, pH 9). Samples were thoroughly mixed and analyzed at least in duplicate.

The percentage inhibition (I%) of soybean lipoxygenase activity was calculated using:

$$I(\%) = \frac{V_C - V_S}{V_C}$$

where V_C and V_S are the initial reaction rates of the control and the

sample, respectively. These rates were determined using the angular coefficient of the fitted equation for the initial portion of the kinetic curve, based on the Michaelis-Menten equation and steady-state assumptions.

The AChE is found at postsynaptic neuromuscular junctions, namely in muscles and nerves, being responsible for the termination of impulse transmissions at cholinergic synapses within the nervous system by rapid hydrolysis of the neurotransmitter, acetylcholine [44]. As there are limited drug options available for its treatment and current therapeutic strategies are mainly cholinesterase inhibitors [45], the AChE inhibitory activity *in vitro* assay was conducted to test the potential activity of *U. tomentosa* leaf extracts (rich in alkaloids Rhy, Imyt, and Myt) against Alzheimer's disease. The isolated alkaloids were also tested. The experiments were performed according to the modified procedure described elsewhere [46], and as previously carried out by the authors [47,48].

Briefly, DTNB (3 mM, 500 μL), AChI (15 mM, 100 μL), Tris–HCl buffer at pH 8 (50 mM, 275 μL), and the extract (previously dissolved in ethanol:Tris-HCl buffer (50:50, v/v), 100 μL) were added to a 1 mL cuvette. The enzyme AChE (0.28 U/mL, 25 μL) was then added to start the reaction, which was monitored for 5 min (25 °C) at 405 nm (UV–vis spectrophotometer, Jasco, Model V650, Japan) for determination of the reaction rate. The AChE activity was calculated as the percentage of this velocity compared to that of the control assay using a buffer:ethanol (50:50) mixture instead of the extract (the inhibitor), and the inhibitory activity was then calculated by subtraction. The blank was performed for each sample and concentration, using all reagents except the enzyme. This reduces the interference of possible colour from *U. tomentosa* extracts in the measured absorbance.

For each sample, the assay was done at least in triplicate, with 3 to 4 concentrations, to calculate the IC_{50} value (concentration of active compound that inhibits 50 % the AChE activity). These concentrations were adapted to the extract based on preliminary assays.

3. Results and discussion

3.1. *U. tomentosa* leaves extraction, alkaloids quantification and enzymatic inhibition

In the preliminary tests, the presence of alkaloids was not detected by TLC (revealed using Mayer's reagent) in the extracts obtained during the first step, where pressurized liquid acetone was employed at 328 K and 15 MPa (data not shown). Although oxindole alkaloids are significantly soluble in acetone [49], Calvo et al. [2] observed that extracts obtained using ethanol or acetone in Soxhlet extraction had significantly lower alkaloid content compared to those obtained using supercritical carbon dioxide modified with ethanol. This may be related to the localization of alkaloids in idioblasts and/or laticifers, cellular structures whose walls may present specific characteristics that hinder, both physically and biochemically, the transfer of these compounds into the solvent [50,51]. Therefore, in addition to solvent selection, it may be necessary to introduce energy into the extraction system, such as mechanical agitation, centrifugal force, microwaves, or ultrasonic waves, to facilitate the release of target compounds into the extracellular medium [52].

Furthermore, Rhy, Myt, and Imyt were not detected in these extracts via UFLC. Compared to Soxhlet extraction results [2], this could be due to a lower content of these alkaloids in the leaves compared to the bark of *U. tomentosa* and/or the effects of high pressure. In the present study, the raw material was extracted using a compressible liquid for 6 h, whereas in Soxhlet extraction, reflux involving evaporation and condensation of acetone occurs over approximately 24 h. Another reason for selecting acetone was its relatively lower polarity compared to ethanol, which could mitigate alkaloid isomerization if they were solubilized during this step [30,31]. Lopez-Avila and Benedicto [25] carried out sequential extraction in plant root, with the first step using pure $scCO_2$ at 25 MPa and 333 K for 0.5 h, followed by the second step at the same temperature and pressure conditions, using $scCO_2$ modified by methanol (10 %) for 1 h. The presence of two and five Myt isomers was observed in the first and second steps, respectively, while Rhy was observed only in the extract obtained in the second step. Therefore, formulated hypotheses should be tested in future studies to consolidate the first extraction step as an effective method for concentrating oxindole alkaloids in the raw material without compromising their integrity.

In the second step of the preliminary tests, indications of alkaloids were observed when CO_2-XLE was used as the solvent at 16 MPa and 333 K. However, no such indication was detected with ethanol at 352 K and 0.1 MPa, even when the analyzed sample amount was 20 times greater than that of CO_2-XLE. To evaluate the recovery of phenolic compounds during the first extraction step, the samples were analyzed by TLC and revealed using NP-PEG, confirming the presence of phenolic compounds in all extracts (data not shown). This suggests that the first step could be optimized to improve the recovery of these compounds; however, it is important to prioritize the integrity of the oxindole alkaloids, which will be extracted in the subsequent step. Consequently, the extraction parameters for the first step were established at 328 K and 15 MPa, while the solvent flow rate and extraction time were determined as described in the previous section.

For the full factorial design results, the acetone extraction step yielded 17 ± 0.8 % (d.b.). For the CO_2-XLE step, the independent factors (X's) and global yield values (Y's) for *U. tomentosa* leaves are shown in Table 1. The global extract yields ranged from approximately 7 % to 10 % (d. b.). The contents of Rhy, Myt, and Imyt varied from 4.34 ± 0.73 to 6.51 ± 0.61, 1.15 ± 0.02 to 2.26 ± 0.03, and 1.23 ± 0.40 to 6.83 ± 0.41 mg of alkaloid per gram of extract (mg/g), respectively.

Considering the sum of Rhy, Myt, and Imyt, the content ranged from approximately 0.6 % to 1.6 %, with five of the six extracts exceeding 1 % (Table 1). These concentrations are comparable to those found in Krallendorn®, although, in this commercial product approximately 97 % of the alkaloids are pentacyclic [2]. In the present study, the ratio of Rhy (a tetracyclic alkaloid) relative to Myt and Imyt ranged from 39 % to 62 % (Rhy:Myt + Imyt ratio), even though Myt is typically the major oxindole alkaloid [4,22,23]. This demonstrates that the selected parameters favoured selective recovery of Rhy, suggesting potential for the development of commercial products from these extracts. Future studies should quantify the total content of other alkaloids and estimate the ratio of tetracyclic to pentacyclic alkaloids.

Regarding the biological potential of the obtained extracts, the inhibition of lipoxygenase and AChE, as well as their correlations with X's and Y's were investigated due to their association with anti-inflammatory and neurological activities, respectively (Table 1, Table 2).

The inhibition of lipoxygenase varied from 12 % to 95 % for extracts at concentrations of 0.5, 1.0, and 2.0 mg/mL obtained by CO_2-expanded liquid ethanol. For the 2.0 mg/mL extract, a strong negative correlation was observed with the Rhy:Myt + Imyt ratio (r = -0.8), while positive correlations were found with T (r = 0.5), P (r = 0.8), global extract yield (r = 0.8), and the total amounts of Rhy, Myt, and Imyt in the extract (r = 0.8). For the 0.5 mg/mL concentration, considerable correlation was observed only with the global extract yield (r = -0.7).

The highest and lowest inhibitions were observed for extracts at 1 mg/mL, with the highest inhibition occurring in the extract obtained at 850 kg/m^3, 313 K, and 27 MPa. At this extract concentration, lipoxygenase inhibition showed strong positive correlations with ρ (r = 0.5) and the Rhy:Myt + Imyt ratio (r = 0.6). However, the highest and lowest inhibitions at this concentration were observed for very similar Rhy:Myt + Imyt ratios (61:39 and 62:38), yet it was observed that despite the higher concentration of Rhy, the concentration of Imyt was approximately half that of the condition with the lowest inhibition (Table 1). This is also evident in the contour plot presented in Fig. 1, which shows the highest inhibitions about the global contents of Rhy and all studied alkaloids (Rhy + Myt + Imyt) in the extracts, as well as extract yield and the Rhy:Myt + Imyt ratio. Furthermore, no correlations

Table 1
Process variables, global yields and contents of rhynchophylline, isomytraphylline and mytraphylline in extracts from *U. tomentosa* leaves.

	ρ (kg/m^3)	T (K)	P (MPa)	Global Yield (%, d. b.)	Rhy (mg/g)	Myt (mg/g)	Imyt (mg/g)	Rhy, Myt and Imyt in extract (%)	Rhy: Myt + Imyt ratio	Lipoxygenase Assay Extract Concentration (mg/mL) 0.5	1.0	2.0	AchE assay IC50 (μg of extract/mL)
1st Step – Acetone	751	328	15	17.0 ± 0.8	n.d.	n.d.	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2nd Step – Ethanol + CO_2	820	313	16	7.36 ± 0.18	6.25 ± 1.02	1.61 ± 0.19	2.60 ± 0.29	1.05 ± 0.15	62:38	54.2 ± 2.1	11.9 ± 0.9	24.4 ± 1.8	1.37 ± 0.02
	850		27	6.83 ± 0.23	4.34 ± 0.73	1.15 ± 0.02	1.23 ± 0.40	0.67 ± 0.11	61:39	61.2 ± 4.2	95.5 ± 3.0	28.4 ± 2.4	0.44 ± 0.21
	870		38	9.56 ± 0.62	6.51 ± 0.61	1.55 ± 0.07	3.56 ± 0.27	1.16 ± 0.03	58:42	22.9 ± 1.2	32.7 ± 0.7	43.35 ± 3.8	1.95 ± 0.42
	770	333	16	8.75 ± 0.15	5.82 ± 0.15	1.92 ± 0.13	5.04 ± 0.40	1.28 ± 0.04	46:54	24.6 ± 1.6	30.5 ± 0.4	36.3 ± 2.3	1.84 ± 0.28
	800		27	8.00 ± 0.01	6.22 ± 0.98	1.59 ± 0.37	4.08 ± 0.33	1.19 ± 0.03	56:44	49.2 ± 4.0	44.9 ± 4.9	29.4 ± 1.6	4.54 ± 0.38
	810		38	9.44 ± 0.21	6.02 ± 0.84	2.26 ± 0.03	6.83 ± 0.41	1.51 ± 0.12	39:61	48.3 ± 2.7	28.05 ± 1.1	56.63 ± 3.3	2.27 ± 0.33

n.d. is not detected. n.a. is not applicable.

Table 2
Linear Correlations for lipoxygenase and AChE assays.

	T (K)	P (MPa)	ρ (kg/m^3)	Global Extract Yield (%)	Global Rhy (mg/g)	Global Myt (mg/g)	Global Imyt (mg/g)	Rhy + Myt + Imyt in Extract (%)	Rhy: Myt + Imyt
Lipoxygenase Assay – 0.5 mg/mL	–	–		−0.7	–	–	–	−0.5	–
Lipoxygenase Assay – 1.0 mg/mL	–	–	0.5	−0.6	−0.8	−0.7	−0.7	−0.8	0.6
Lipoxygenase Assay – 2.0 mg/mL	0.5	0.8		0.8	–	0.7	0.8	0.8	−0.8
AChE Assay IC_{50}	0.7	–	–	–	–	–	0.5	0.5	−0.5

were observed with X's and extract yield at this concentration, which facilitates attributing the inhibitory effects to the quantities and Rhy: Myt + Imyt ratio.

Additionally, it should be taken into account that each lipoxygenase has its specificity, and the performance of an inhibitor may vary with the enzyme [43,53]. The anti-inflammatory activity of *U. tomentosa* is also related to the presence of other compounds besides alkaloids such as Myt, as previously verified by other authors [54].

The AChE inhibitory activity assay was carried out to infer the possible *in vitro* anti-Alzheimer's potential of *U. tomentosa* extracts rich in oxindole alkaloids. Results are expressed as IC_{50} values (Table 1). These experiments revealed that the buffer:ethanol (50:50, v/v) solution did not inhibit the enzyme, and therefore it was used to solubilize both extracts and standards (Rhy, Myt, and Imyt). For extracts obtained at 313 K, the IC_{50} ranged from ~ 0.4 to 2.0 mg/mL, while for extracts at 333 K, IC_{50} values were, in general, higher, ranging from ~ 1.8 to 4.5 mg/mL. This strong positive correlation to have higher IC_{50} values or lower AChE inhibitions ($r = 0.7$) at higher temperature (Table 2) may be due to the loss of some bioactive thermo-degradable compounds at 333 K. For all extracts, a linear response ($0.8758 \leq R^2 \leq 0.9999$) was observed for the AChE inhibition as a function of tested concentrations. Regarding the standards, it was not possible to calculate an IC_{50} value because the tested concentrations showed AChE inhibitory activity lower than 50 %. Nevertheless, based on those results, IC_{50} values were estimated: 7.37 ± 2.02 mg/mL for Rhy, 5.33 ± 0.08 mg/mL for Myt, and 36.20 ± 4.84 mg/mL for Imyt. Despite estimated IC_{50} values, the ability of these isolated alkaloids to inhibit the AChE enzyme was lower than that of the extracts rich in those compounds. This means that the anti-Alzheimer potential of extracts appears to be due to other components or to a possible synergistic effect of alkaloids/ other compounds [55,56]. A strong negative correlation was observed between IC_{50} values and the Rhy:Myt + Imyt ratio in the extracts (Table 2). Lower IC_{50} values were associated with lower concentrations of Rhy relative to Myt and Imyt, as shown in Fig. 1. Similar results were observed in the lipoxygenase inhibition assays further emphasizes the significance of alkaloid proportions. This type of alkaloid, namely Rhy, appears to have a neuroprotective effect, including in Alzheimer's disease [14,18–20].

Other authors have already observed AChE inhibition by *Uncaria* species (*U. rhynchophylla* stem), with values close to 80 % for an extract concentration of 0.1 mg/mL. This higher inhibition value may be due to a different extract composition, namely, related to the presence of catechin, as shown by the molecular docking analysis [45]. Aqueous bark extracts of *U. tomentosa* have also revealed almost 80 % inhibition at 0.4 mg/mL ($IC_{50} = 0.112$ mg/mL), which may be associated with the presence of oxindole alkaloids [13]. Distinct degrees of inhibition may be related to different extraction conditions, plant species, and plant harvesting regions, among others, and comparisons are not straightforward. Finally, the results presented in this study indicate that maximizing the content of Rhy and minimizing Imyt may lead to significant inhibitory activity against lipoxygenase and AChE.

3.2. Process modelling, optimization and stability

To comparatively evaluate the effects of X's on Y's and perform an initial screening of X's effects for the construction of the mathematical model, pairwise correlation and PCA methods were used. From the pairwise correlation analysis (Table 3), it was observed that ρ seems not influence the total extract yield and content of Rhy in the extract. However, strong negative correlations were observed for Myt and Imyt, as well as a strong positive correlation with Rhy:Myt + Imyt ratio. Regarding P, weak correlations were observed with all Y's. Except for Imyt and Rhy:Myt + Imyt ratio, t showed strong correlations with the other Y's, indicating that the recovery of Imyt and higher values of Rhy: Myt + Imyt ratio occur predominantly at the beginning of extraction or remain constant along the process. Regarding T, no correlation with extract yield was observed, whereas for Myt and Rhy:Myt + Imyt ratio, correlations of 0.57 and −0.69 were observed, while for Imyt, the correlation was 0.73. Despite the lack of correlation with extract yield, the

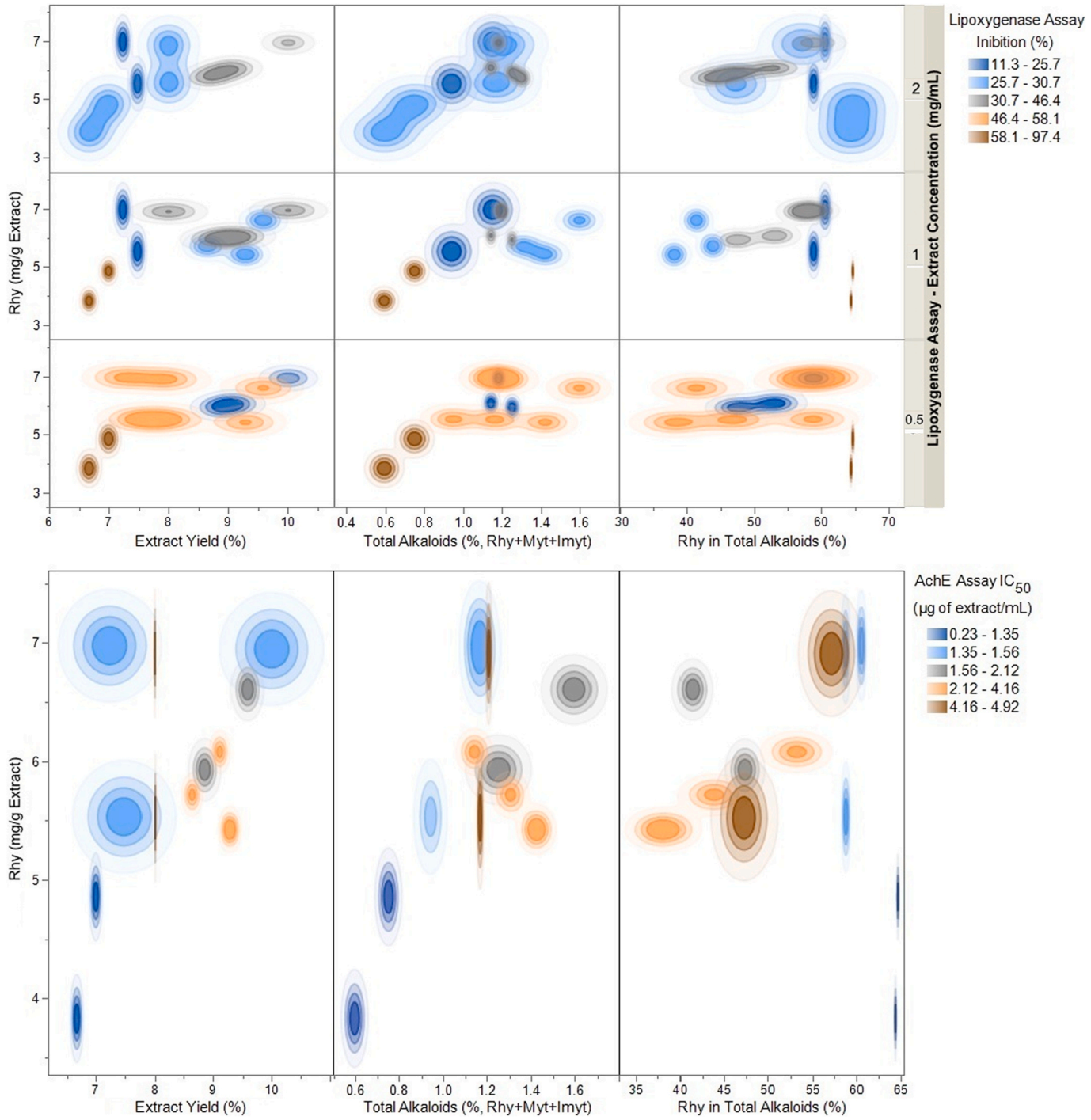

Fig. 1. Lipoxygenase and AChE assays contour plots related to alkaloid contents in *Uncaria tomentosa* extracts.

Table 3
Correlations for extract yield, alkaloids contents (Rhy, Myt, Imyt), and extraction process conditions (T, P, ρ and t).

	T (K)	P (MPa)	ρ (kg/m^3)	t (min)
Extract Yield (%)	0.06	0.19	0.04	0.82
Rhy (mg/g)	0.41	−0.06	−0.40	0.48
Myt (mg/g)	0.57	0.05	−0.50	0.47
Imyt (mg/g)	0.73	0.22	−0.54	0.17
Rhy in Rhy + myt + Imyt (%)	−0.69	−0.21	0.50	−0.13

correlations for the alkaloids may be related to hydrogen bonding, van der Waals forces, and dipole–dipole interactions, which can be disrupted by increasing temperature. Thus, an increase in temperature may reduce the diffusion activation energy for the desorption of the solute from the matrix, improving the diffusion and solubility of the solute from within the particles to the extractive medium [57]. Furthermore, T may have influenced the isomerization of Myt. These correlations can be visualized in the extraction kinetic curves, where the variations in alkaloid concentrations over time are also observed. The alkaloid contents did not show significant differences for the various combinations of X's; however, it was noted that at 770 and 810 kg/m3, the Rhy and Imyt contents exhibited similar extraction profiles and quantities (Fig. 2). The

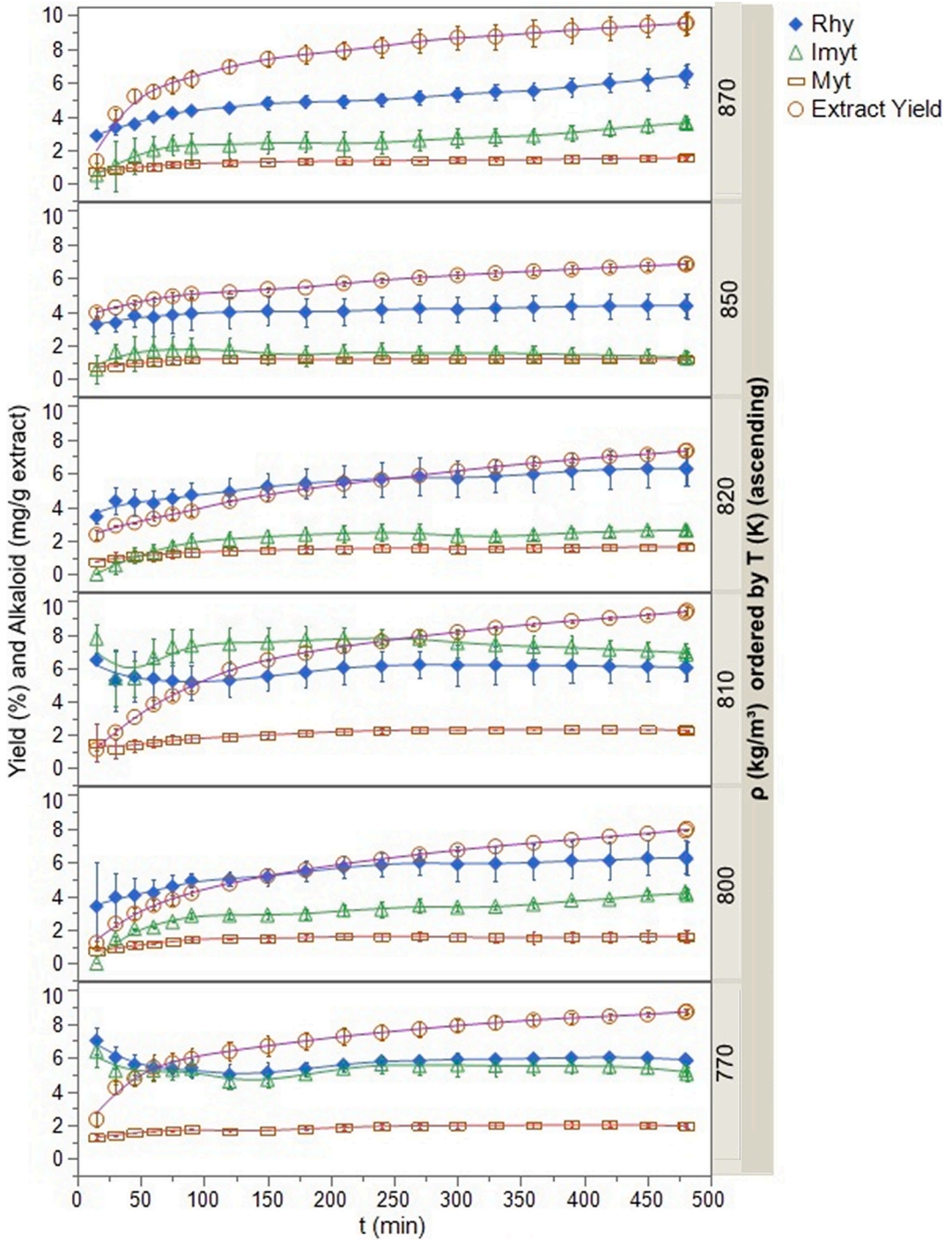

Fig. 2. Overall extraction curves for the extract yield of *Uncaria tomentosa*, alkaloids contents (Rhy, Myt and Imyt) according to extraction process conditions of temperature (T) and solvent density (ρ).

lower contents of Myt compared to Imyt may be related to isomerization reactions occurring during the extraction process. Although CO_2 can act as a Lewis acid or base [29], the lipophilicity of this solvent may facilitate the isomerization reactions of oxindole alkaloids from *U. tomentosa* [30,31]. Additionally, the use of CO_2 as a co-solvent (25 % w/w) in pressurized liquid ethanol results in a gas-expanded ethanol solvent with improved transport properties, which can solvate the oxindole alkaloids due to their solubility in ethanol. Kaiser et al. [27] observed that reflux extraction, turbo-extraction, and static maceration were processes that led to greater isomerization of oxindole alkaloids, whereas ultrasound-assisted extraction and dynamic maceration at relatively low temperatures (~23 °C) and short extraction times (45 min to 2 h) resulted in less isomerization.

From the PCA, where the Y's were reduced to two components that accounted for 86.8 % of the variance in the data, these components represented linear combinations of extract yield and the contents of Rhy, Myt and Imyt. Among the evaluated parameters, T was identified as the most influential. For example, the combination of results obtained at 313 K and 870 kg/m3 can be considered significantly different from the combination of results obtained at 333 K and 810 kg/m^3, as the data appear in the outer regions of the cluster (Fig. 3).

Based on the above observations, Y's were modelled with ρ, T, and t.

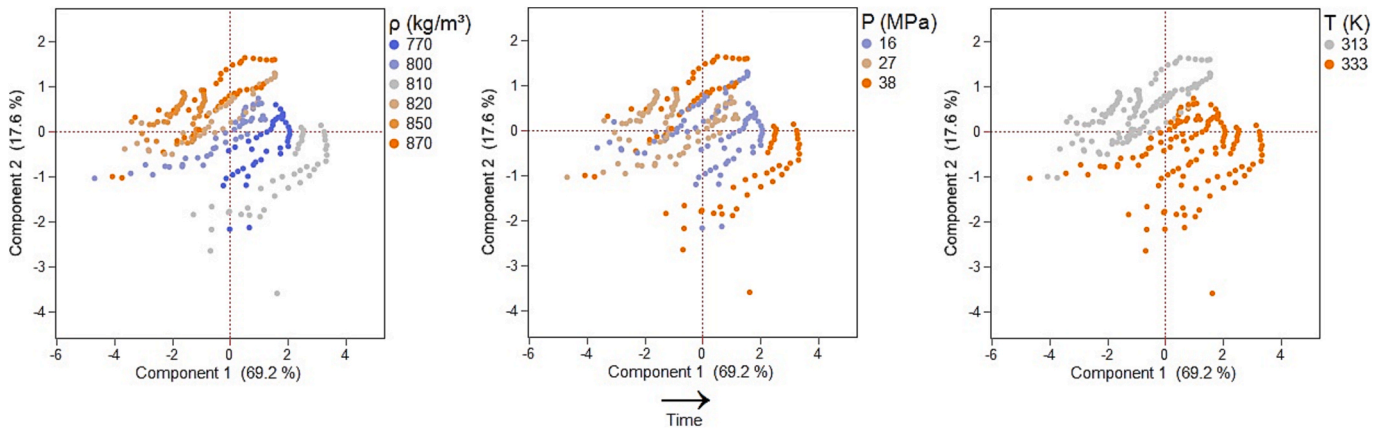

Fig. 3. PCA plot for extract yield, alkaloids contents (Rhy, Myt, Imyt) colored by density(ρ), pressure (P) and temperature (T). Each principal component represents a linear combination of extract yield and alkaloid contents. The percentage values indicate the proportion of total data variance explained by each component.

The model effects were estimated by fitting the data to both full factorial and polynomial models up to the fourth order. The fitting method used was least squares. The best fit was obtained with a polynomial that showed a quadratic effect on ρ and t. The statistical parameters for evaluating the model were similar for both fitting and validation. These parameter values are presented in Table 4. Regarding Rhy prediction, the similarities between the statistical parameters for fitting and validation, along with a significant *p-value* and an insignificant lack of fit, indicate that the fitting is reasonable, despite an R^2 value of 0.57. These parameters suggest that despite high standard deviations, this alkaloid can be predicted with reasonable confidence, as observed in Fig. 4, where the grey shadow illustrates the 95 % of confidence region for individual predicted values. Therefore, the predicted values for Rhy should have a larger confidence interval than the other Y's, and thus, in the optimization study, the values of X's where the confidence interval for the Rhy prediction is smaller were also considered.

The optimization study of the X parameters was conducted based on piecewise smooth functions and Bayesian optimization methods. Since pentacyclic oxindole alkaloids are predominant in the extracts reported in the literature and commercial products, the optimization of parameters was based on maximizing the Rhy content and minimizing Myt and Imyt in the extract. Since extract yield is strongly correlated with t, and to avoid conditioning the study based on its effect, extract yield was included in the optimization study as a neutral parameter for minimization or maximization effects in the optimization platform in JMP. For similar reasons, extract yield was not included in the objective function construction for analysis in GPyOpt. Furthermore, for the construction of the objective function, the concentrations of Rhy, Myt, and Imyt were first converted into a principal component (77.6 % of variation explained) by PCA. Then, this component was adjusted for the X's (Table 4). To obtain the optimized parameters, 200 iterations were performed, and the X's range values were also considered. The optimized parameters can be consulted in Table 5. For both methods, optimized ρ values were obtained for 15 min of extraction at 313 K.

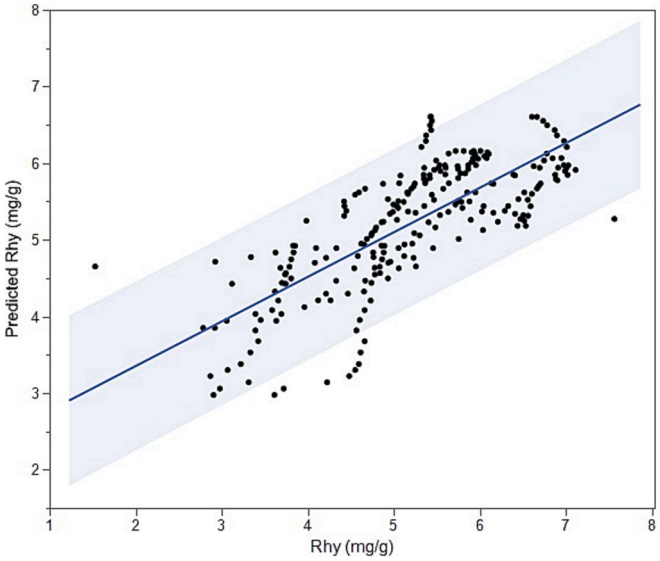

Fig. 4. Rhy content prediction versus obtained data. Grey area indicates the individual 95% confidence interval of prediction.

Table 4
Mathematical model parameters and effects for extract yields and contents of Rhy, Myt and Imyt in extract.

Terms	Contribution to Fit Least Square Model (LogWorth)	Dependent Parameters/Principal Component/Statistical Parameters for Model				
t	100.04					
T	42.89	Statistical Parameter	Extract Yield (%)	Rhy (mg/g)	Myt (mg/g)	Imyt (mg/g)
ρ^2	22.07					
t^2	21.85	R^2	0.90	0.57	0.75	0.68
T × ρ	19.66	Lack of Fit	<0.0001	0.9529	0.0009	<0.0001
ρ	12.01	RMSE	0.6548	0.7309	0.2072	1.2192
T × t	7.29	*p*-value	<0.0001	<0.0001	<0.0001	<0.0001
ρ × t	3.06					
T	26.29	Statistical Parameter		Principal Component		
t^3	22.24	R^2		0.73		
ρ^2	15.80	Lack of Fit		0.0005		
T × ρ	15.39	RMSE		0.8017		
t^2	7.92	*p*-value		<0.0001		
T × ρ^2	2.20					

Table 5
Estimated optimized independent parameters and simulated control limits and process stability index.

		Piecewise smooth functions		Bayesian optimization	Contour plot	Robustness (Flatness)
T (K)		313	313	313	313	313
P (MPa)		23	28	29	28	26
ρ (kg/m^3)		835	847	849	848	842
t (min)		15	15	15	36	481
Predicted Rhy (mg/g)		3.29 ± 0.52	2.97 ± 0.52	2.97 ± 0.52	3.09 ± 0.33	4.88 ± 0.40
Predicted Myt (mg/g)		0.72 ± 0.15	0.61 ± 0.14	0.61 ± 0.14	0.69 ± 0.8	1.08 ± 0.11
Predicted Imyt (mg/g)		0.05 ± 0.47	0.00 ± 0.26	0.00 ± 0.29	0 ± 0.46	0.65 ± 0.66
Predicted Extract Yield (%)		2.47 ± 0.47	2.71 ± 0.46	2.80 ± 0.46	3.17 ± 0.30	6.55 ± 0.35
Rhy: Myt + Imyt		81:19	83:17	83:17	82:18	74:26
Rhy	UCL (mg/g)	5.23	5.15	5.23	5.26	7.08
	LCL (mg/g)	1.06	0.79	0.74	0.91	2.70
	Limit Range	4.17	4.36	4.49	4.35	4.38
	Stability Index	0.98	1.00	0.99	1.01	0.99
Myt	UCL (mg/g)	1.34	1.23	1.23	1.31	1.70
	LCL (mg/g)	0.09	0	0	0.08	0.46
	Stability Index	0.99	0.99	1.00	1.01	0.99
Imyt	UCL (mg/g)	3.76	3.41	3.46	3.62	4.39
	LCL (mg/g)	0	0	0	0	0
	Stability Index	0.99	1.00	1.00	0.99	0.99
Extract Yield	UCL (%)	4.43	4.70	4.78	5.11	8.54
	LCL (%)	0.55	0.73	0.82	1.24	4.58
	Stability Index	1.01	0.99	1.01	1.00	0.99

UCL is upper control limit. LCL is lower control limit.

However, for the piecewise smooth function-based method, two ρ values were found: 835 and 847 kg/m^3 for maximizing Rhy content and minimizing Myt and Imyt. On the other hand, for Bayesian optimization, the optimized ρ was 849. These results show that although a deterministic method and a stochastic method were used, both indicate the optimized parameters at 313 K, ~848 kg/m^3, and 15 min. Another alternative for finding the optimized parameters is the contour plot (JMP), where the regions where Myt and Imyt are minimized can be explored, with this region represented by the white area in the contour plot presented in Fig. 5. This region was delineated by the estimated minimum values of 0.7 mg/g for Myt and 0 mg/g for Imyt. Thus, it is possible to observe that these minimization conditions are achievable from 313 K up to 36 min of extraction. Another option for optimizing the parameters is the robustness study, where the partial derivative was applied to the least squares fitted model concerning ρ, as T and t are relatively easy to control, while ρ may experience slight variations due to solvent pressure control. The derived equations obtained were solved for values close to 0, using the piecewise smooth function method. The estimated optimized parameters can be consulted in Table 5.

To estimate the control limits and stability of the process relative to the optimized parameters discussed above, 10,000 Y's were simulated using the Monte Carlo method (JMP). The predicted values were used to estimate the stability index, UCL, and LCL (Table 5). The stability index values were all approximately one, indicating that the simulated results suggest a stable process. When examining the control limits ranges, it was observed that the range for Rhy content obtained at 313 K, 835 kg/m^3, and 15 min was slightly smaller, though this difference represents about 5.5 % when compared to the second smallest range estimated (313 K, 848 kg/m^3, and 36 min) and the average estimated Rhy content. For Rhy obtained at 313 K, 835 kg/m^3 and 15 min, the largest range was

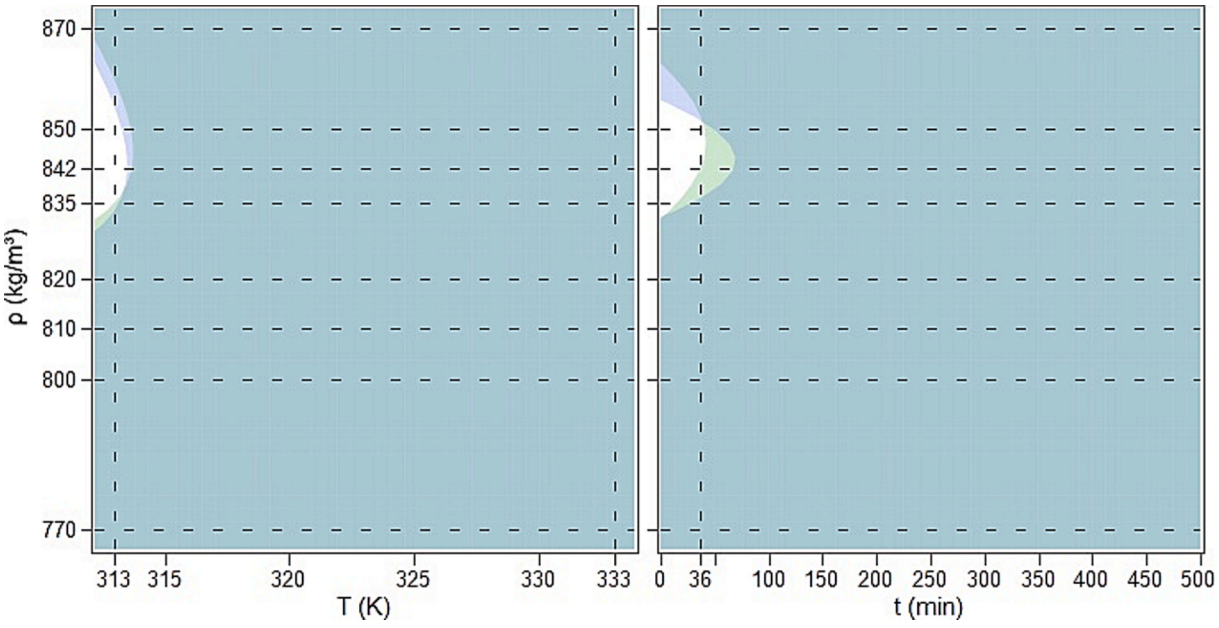

Fig. 5. Contour plots for maximization of Rhy content related to solvent density (ρ), temperature (T) and process time (t).

observed for Imyt, with UCL between 3.41 and 4.39 mg/g, indicating that this process should be optimized in future studies to mitigate variations in Imyt and Rhy to maintain Rhy as the major compound and ensure minimal variability. Based on the role of traditional and complementary medicine in healthcare systems [58], future studies should include a broader analytical screening of the extracts to better define their composition for possible optimization and/or application in clinical or preclinical trials, following guidelines for the preparation and characterization of extracts for pharmacological and toxicological applications [59], including the quantification of isorynchophylline to assess potential isomerization effects of Rhy.

For the optimized parameters mentioned above, the Rhy:Myt + Imyt ratio was estimated to range from 74 % to 83 % relative to the total content of these alkaloids (Table 5) for extraction times of 15, 36, and 481 min, with the minimization of Myt and Imyt being statistically more favourable for extraction times between 15 and 36 min. However, the extract can be utilized in varying ratios of these alkaloids. Fig. 6 shows the extract yield and the Rhy:Myt + Imyt ratio in the collected extract throughout the extraction process. Despite the correlations observed between the concentration of Rhy in the accumulated extract and T (0.41) and ρ (−0.40), a significant strong negative correlation (−0.69) was found between T and the Rhy:Myt + Imyt ratio and strong positive correlation for ρ (0.50) and the Rhy:Myt + Imyt ratio for extract fractions evaluated individually over time (Table 3). No correlation was observed between t and the proportion of Rhy relative to Myt and Imyt, suggesting that the solubility of these compounds remains relatively constant over time for each extraction. This stability may be attributed to the low variation in extract mass at each time point.

Finally, the optimization and process performance studies of the extraction parameters indicate a feasible control of alkaloid content, particularly maximizing Rhy, while minimizing Myt and Imyt under 313 K and different ρ and t. The robust correlation between ρ with alkaloid concentrations highlights the importance of precise control over these factors during extraction. The predictive modelling and Monte Carlo simulations further support the stability of the process, providing a reliable framework for optimizing extraction protocols. Future work should focus on more optimization studies on these conditions to reduce variability, ensure consistent extraction profiles and enhance the quality of the final product for potential pharmaceutical applications.

4. Conclusions

This study highlights the potential of *U. tomentosa* leaves as a rich source of oxindole alkaloids, particularly Rhy, which was optimized to achieve higher proportions relative to the sum of Myt and Imyt. The extracts demonstrated promising inhibitory effects on lipoxygenase and acetylcholinesterase, indicating potential anti-inflammatory and neuroprotective properties. Sequential extraction was shown to effectively enhance the recovery of oxindole alkaloids by maximizing the removal of non-oxindole compounds in the initial step. The use of CO_2-expanded liquid ethanol in the second step allowed for the optimization of parameters, such as T and ρ, to significantly improve the Rhy:Myt + Imyt ratio. Optimal extraction conditions of 313 K, ~848 kg/m^3 solvent density, and 15–36 min extraction time were identified to maximize Rhy content while minimizing Myt and Imyt concentrations in the extracts.

Furthermore, advanced data analysis employing deterministic and stochastic approaches supported the development of a robust, stable, and controlled extraction process. However, the variability in alkaloid content and potential synergistic effects among compounds warrant further investigation in future studies to optimize the formulation for product development, adhering to phytotherapeutic efficacy guidelines.

Finally, the use of *U. tomentosa* leaves addresses waste management concerns by repurposing a high-alkaloid-content byproduct, thereby promoting sustainable practices in phytotherapeutic production and enabling the extraction of different alkaloid ratios for potential diverse biological applications.

CRediT authorship contribution statement

José Rafael S. Botelho: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Marisa C. Gaspar:**

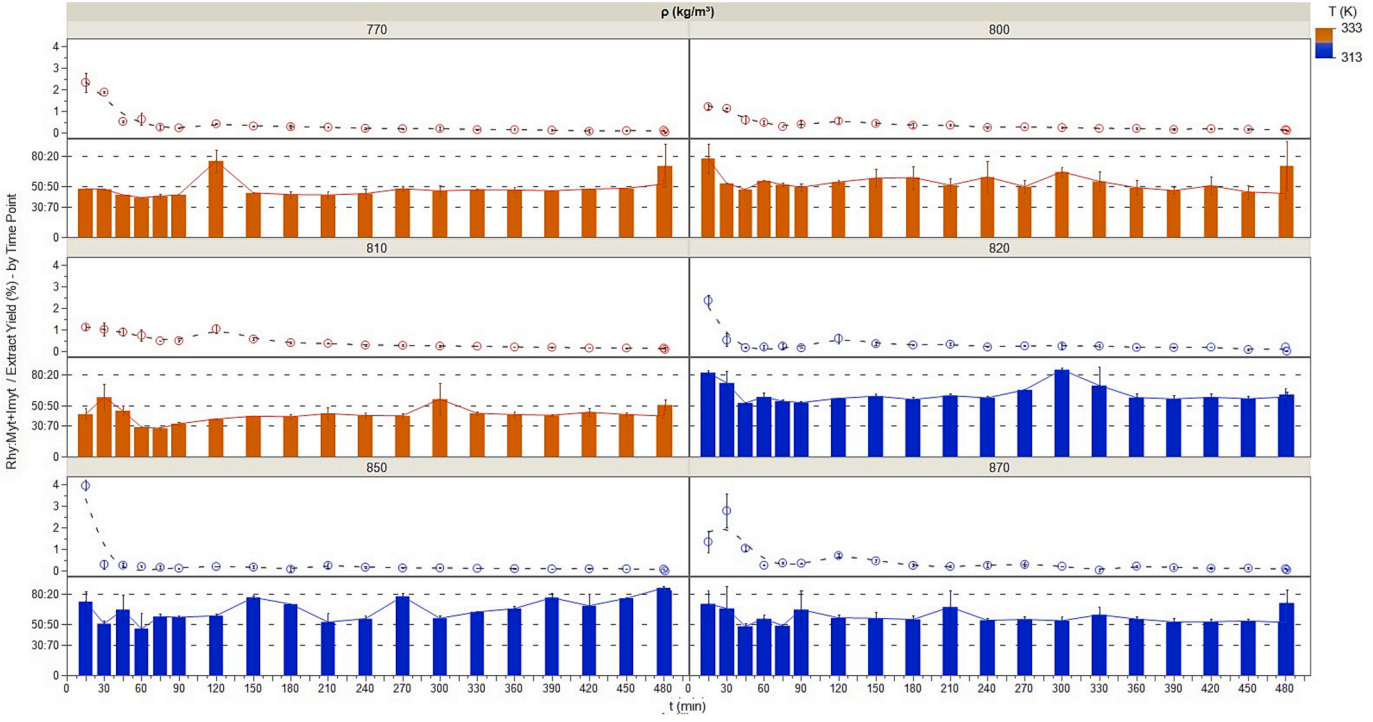

Fig. 6. Kinetic extraction yield (%) represented by circular symbols and Rhy content ratio (Rhy: Myt + Imyt) in the extracts in function of solvent density (ρ), temperature (T).

Writing – review & editing, Investigation. **Hermínio J.C. de Sousa:** Writing – review & editing, Visualization, Supervision, Resources. **Mara E.M. Braga:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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