Quality-by-Design development of Celtis iguanaea (Jacq.) Planch (Cannabaceae) microparticles with in vivo gastroprotective efficacy

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Abstract

Celtis iguanaea, widely used in Brazilian folk medicine, is known for its gastroprotective properties. This study aimed to develop a spray-dried hydroethanolic leaf extract of *C. iguanaea* (SDCi) and evaluate its gastroprotective efficacy in mice. A Box-Behnken design with three factors at three levels (n = 15) and Response Surface Methodology (RSM) were used to optimize the spray drying. The variables studied were drying air inlet temperature (IT, 100–140°C), extract feed flow rate (Ef, 0.3–0.7 L/h), and leucine content (Lc, 15–45% m/m relative to solids) as a drying adjuvant. The IT and Lc were the main factors affecting the drying process. Optimal conditions were IT of 120°C, Ef of 0.3 L/h, and Lc of 45%, achieving a yield of 52.0% with microparticles of 3.4 µm. However, energy efficiency requires improvements. The optimized SDCi contained 21.8 mg/g total polyphenols, 49.7 mg/g flavonoids, and 518.8 mg/g phytosterols, with IC_{50} of 301.6 µg/mL and electrochemical index of 6.1 µA/V. LC-PDA, LC-UV, and ESI(-)-MS analyses identified hydroxycinnamic acid and two flavones as major compounds. In an indomethacin-induced gastric ulcer model, SDCi (150 and 300 mg/kg, p.o.) significantly reduced the lesion index, potentially due to its immunomodulatory effects, including decreased IL-1β and increased IL-10 levels in the stomach. This QbD approach successfully developed a high-value phytopharmaceutical intermediate product with favorable yield, antioxidant properties, and *in vivo* gastroprotective efficacy. Further studies on stability, pharmacokinetics, drug interactions, and human safety are needed to support its use in Brazilian complementary medicine.

Introduction

Peptic ulcer disease (PUD) stands out as one of the most clinically relevant gastrointestinal disorders. Recent studies demonstrated that the prevalence of this illness increased by approximately 25.7% globally over thirty years (1990–2019) (Ren et al., 2022; Xie et al., 2022). The causes commonly associated with PUD include *Helicobacter pylori* infection and the prolonged or indiscriminate use of nonsteroidal anti-inflammatory drugs (NSAIDs, *e.g.*, indomethacin, naproxen, diclofenac, etc.). Other factors such as genetics and environmental influences (*e.g.*, stress, smoking, diet, and alcohol consumption) also play a role (Almadi et al., 2024; Bereda, 2022). The pharmacological treatment may involve *H. pylori* eradication therapy with antibiotics (*e.g.*, clarithromycin, amoxicillin, metronidazole, levofloxacin, and bismuth) and the use of H_2-receptor antagonists (*e.g.*, cimetidine and ranitidine) or proton-pump inhibitors (*e.g.*, esomeprazole and omeprazole). However, the side effects of these drugs can impact patient adherence to treatment and, consequently, reduce its efficacy (Almadi et al., 2024).

Driven by the challenges of developing more effective and safer therapeutic options for managing PUD, our research team has been exploring the valuable traditional knowledge and remarkable biodiversity of the Brazilian Savanna (Cerrado) as a source of gastroprotective herbal medicines over the past few years (da Silva et al., 2016; José et al., 2017; Martins et al., 2015; Rosa Júnior et al., 2024). Among the various species studied so far, *Celtis iguanaea* (Jacq.) Planch. (Cannabaceae), popularly known as “esporão-de-galo” in Portuguese, stands out for its safety (Borges et al., 2013; Gonçalves et al., 2015; Haas da Silva et al., 2016) and preclinical gastroprotective and antisecretory efficacy demonstrated in several animal models (De Sousa et al., 2013; Martins et al., 2014a, b). We were the first to elucidate the underlying mechanisms of gastroprotective action, including increased mucus adhesion to the gastric wall and the involvement of α2-adrenoceptors, nitric oxide (NO), and prostaglandins. These effects are likely associated with hydroxyl-linolenic acid, linoleic acid, and conjugated oxo-linoleic acids that were identified in the leaf extract (Martins et al., 2014a).

The next step toward developing high-value phytopharmaceutical products from *C. iguanaea* leaf extracts is to fully understand its behavior during unit operations, such as spray drying, which is widely used in the food and pharmaceutical industries worldwide to obtain dried powders from heat-sensitive materials (Banožić et al., 2023; Donthi et al., 2024). Spray-drying is a critical process in the development of phytopharmaceuticals, as it directly impacts the stability, morphology, flowability, dissolution, and, consequently, the suitability of the dried product for solid pharmaceutical dosage forms (de Oliveira et al., 2023; Gallo et al., 2015; Gallo and Bucalá, 2019; Martins et al., 2020). These bring several advantages over liquid extracts regarding shelf-life, logistics, dosage accuracy, prescription, and posology (List and Schmidt, 1989; Oliveira et al., 2006; Sharapin et al., 2000). Notably, the drying stages while producing phytopharmaceutical products have a significant economic impact on the associated costs, accounting for 50–90% of the total processing costs. Therefore, drying methods must be effective in terms of production volume and maintaining the product's characteristics within the desired quality standards, while also being energy-efficient (Belwal et al., 2022).

Quality by Design (QbD) is a strategic approach widely employed in industries to ensure that products are consistently designed and produced to meet predefined quality standards (Jeong et al., 2024; S. H. Lee et al., 2022; Simão et al., 2023). In the context of spray drying, QbD offers a framework to balance product quality, sustainability, and cost-efficiency. By incorporating QbD principles, industries can optimize the spray drying process, ensuring that the final product meets desired specifications besides being produced with minimal resource usage and operational costs (Henriques et al., 2021). Among the various QbD approaches, the Design of Experiments (DoE) and multivariate statistical analyses, such as Response Surface Methodology (RSM), stand out as key tools. DoE allows for the systematic evaluation of the effects of multiple variables on the desired product characteristics (Ziaee et al., 2019), while RSM helps identify optimal conditions for the spray-drying process by modeling the relationship between input factors and output responses (Homayoonfal et al., 2024). These methodologies enable the development of robust, high-quality products with enhanced process understanding, besides ensuring efficiency and cost-effectiveness (Sutar and Yadav, 2023).

This study employed a Box-Behnken DoE and RSM to systematically evaluate the effects of key in-process parameters—drying air inlet temperature, extract feed flow rate, and drying aid content—on the spray drying of *C. iguanaea* hydroethanolic leaf extract. Additionally, the gastroprotective efficacy of the optimized dried extract was evaluated in mice. Our findings contribute to the growing body of evidence supporting the traditional use of *C. iguanaea* in Brazilian folk medicine for managing NSAID-related PUD.

Material and Methods

Chemicals and Reagents

Ranitidine (25 mg/mL injectable solution) from Laboratório Teuto Brasileiro S.A. (Anapólis, GO, Brazil); β-sitosterol, Tween® 80, rutin, and DPPH from Sigma Aldrich Co. (St. Louis, MO, USA); ethanol from Synth® (Diadema, SP, Brazil); gallic acid and HPLC-grade methanol from Êxodo Científica (Sumaré, SP, Brazil); chloroform from Cromato (Diadema, SP, Brasil); Na_2CO_3 from Sciavicco (Belo Horizonte, MG, Brazil); $AlCl_3$ from Neon (Suzano, SP, Brazil); Folin-Ciocalteu reagent and acetic anhydride from Dinâmica (Indaiatuba, SP, Brazil); leucine from Jiangsu Xinhanling Biological Engineering (Xinyi City, Jiangsu, China), colloidal silicon dioxide (CAB-O-SIL®) from Cabot Corporation (Boston, MA, USA), mannitol from Cromoline Ltda. (São Paulo, SP, Bazil), corn starch from Infinity® Pharma (Campinas, SP, Brazil), and microcrystalline cellulose (Avicel®) from IFF Pharma Solutions (Sydney, Australia) were used in this research. Ultrapure water was obtained from a PURELAB® Option Q system (ELGA® LabWater, Lane End, UK), All the other chemicals were of analytical grade.

Herbal Material

Access to genetic heritage was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the code A908B3F. *C. iguanaea* leaf samples were collected from the third and fourth nodes of individuals growing in a modified “Cerrado” region in the rural area of São Sebastião do Oeste, Minas Gerais, Brazil (20°16'30.9"S – 44°56'40.8"W and 20°19'21.2"S – 45°03'24.1"W) on March 27 and April 2, 2022, at the beginning of Brazilian fall. Dr. Andréia Fonseca Silva carried out the botanical identification, and voucher specimens were deposited at the Herbarium of the Agricultural Research Company of Minas Gerais (EPAMIG) under registration number PAMG 59090. The leaves were dried in an oven with natural air circulation (< 40°C) until constant weight and then powdered using a Willey-type knife mill to obtain the plant material from *C. iguanaea* leaves.

Obtaining and Standardization of Feeding Extract

The *C. iguanaea* hydroethanolic leaf extract (HECi) was obtained by percolating 2 kg of plant material using a 70% hydroethanolic mixture (m/m) as the solvent. The percolation flow rate was 0.7 mL/min (BRASIL, 2019). Thin-layer chromatography coupled with ultraviolet/visible detection was used to monitor the leaching of bioactive compounds (i.e., polyphenols, flavonoids, and phytosterols) from the plant material during the extraction process. For polyphenol detection, elution was performed with ethyl acetate:acetic acid:water (100:11:10). The revelation was done by spraying the chromatographic plate with a natural products

reagent (1% diphenylborinoxyethylamine in methanol), followed by polyethylene glycol 4000 in 5% ethanol and reading at 365 nm (Wagner and Bladt, 1996). For terpene detection, the eluent was toluene:ethyl acetate (93:7), and the revelator was 1% vanillin in ethanol, followed by a 10% ethanolic solution of sulfuric acid and heating at 100°C for 10 minutes (Wagner and Bladt, 1996). The extraction process was terminated when none of these markers were detected in the chromatographic analyses. Afterward, HECi was concentrated in a rotary evaporator RV 10 digital (IKA® do Brasil, Campinas, SP, Brazil) under reduced pressure (-600 mmHg) at a temperature lower than 40°C to adjust the solid and ethanol content to levels suitable for the spray drying, ensuring satisfactory yield and safety.

The HECi was characterized in terms of solid content (Sc, % m/m), apparent density (ρ), extraction yield (EY, % m/m), g/mL), pH, total phenolic content (TPc, mg/g; gallic acid equivalent), total flavonoid content (TFc, mg/g; rutin equivalent), reducing capacity expressed by the half-maximal inhibitory concentration (IC_{50}, µg/mL) of DPPH, and electrochemical index (EI, µA/V). Further experimental details are provided in the Supplementary Material.

Spray Drying

Equipment

For the drying of HECi and obtaining the various SDCi, a spray dryer model MDS 1.0 (Labmaq, Ribeirão Preto, SP, Brazil) was used, featuring a co-current flow regime, an evaporation capacity of up to 1.0 L/h, and a maximum operating of 180°C. The system consists of a peristaltic pump, a dual-fluid top pneumatic atomizer with an internal diameter of 1.2 mm, and a cylindrical drying chamber with a 160 mm diameter and 645 mm length, made of polished stainless steel with a sanitary finish. The drying air was supplied by a compressor, electrically heated, with the temperature regulated by a digital thermostat.

Preliminary Drying Studies

Pilot tests were conducted using an inlet drying air temperature of 120°C and an extract feed flow rate of 0.5 L/h. The extract (SDCi0) was evaluated for drying yield (DY, % m/m), TPc, TFc, and IC_{50}. Due to the very low powder recovery and high hygroscopicity of the SDCi0, the use of a drying adjuvant was deemed necessary. Colloidal silicon dioxide, mannitol, microcrystalline cellulose, corn starch, and leucine were tested as drying aids. HECi was mixed with 15% (m/m, based on the solid content of the extract) of each drying adjuvant for three hours and then a sample (5 g) was poured into Petri dishes. The mixture was dried overnight at 100 ± 5°C. The macroscopic appearance of the dried material and its adherence to the plate were evaluated to select the most suitable adjuvant.

DoE

A Box-Behnken experimental design with three levels and three factors (3^3) with three central point replicates (n = 15) (Box et al., 1978) was used to evaluate the effects of drying air inlet temperature (IT, ºC), extract feed flow rate (Ef, L/h), and leucine content (Lc, % m/m relative to the solid content) and optimize the properties of the spray-dried extracts. Table 1 shows the DoE matrix and the coded and uncoded levels of the studied independent variables. The experiments were randomized to minimize experimental bias. These factors were coded to enable Analysis of Variance (ANOVA) by the RSM according to the coding rule defined by Eq. 1:

$$Coded\ value = \frac{Uncoded\ value - 0.5 \times\ (high\ level + low\ level)}{0.,5 \times\ (high\ level - low\ level)}$$

1

Throughout the experimental runs, the drying air flow rate was set at 405.0 L/min, the atomization air flow rate at 40 L/min, the dryer feed flow rate at 0.6 L/h, the compressed air pressure at 3.5 kgf/cm², and the mass of the feed extract (Em) at 100 g. After establishing the experimental conditions, the drying processes were initiated with distilled water to achieve thermal equilibrium. The several dried extracts were separated from the air with a stainless-steel cyclone, collected in a borosilicate glass container,

weighed, and stored in sealed flasks, sheltered from light and moisture, in desiccators at room temperature (24 ± 2ºC) before and after their characterization.

Quality Attributes of the Drying Process

The evaluated responses were DY, moisture content (Mc, % m/m), TPc, TFc, total phytosterol content (TPSc, mg/g; β-sitosterol equivalent), IC_{50}, EI, initial drying air outlet temperature (Ti, ºC), final drying air outlet temperature (Tf, ºC), and energy efficiency (η, %). Moreover, Scanning Electron Microscopy (SEM) analysis revealed the morphological features; and HPLC-DAD, HPLC-UV, and ESI-(-)-MS analysis identified the major phytochemicals of the optimized product. Further experimental details are provided in the Supplementary Material. All responses were coded according to Eq. 1, ranging from − 1 to 1, to ensure they had the same order of magnitude.

Comparing Spray and Freeze-drying

Building on the findings from the preliminary (SDCi0) and optimized (SDCi11) studies, further experiments were conducted to evaluate the impact of the drying method (spray drying and freeze-drying) and Lc (0% and 45% m/m based on the extract's solid content) on the TPc, TFc, and TPSc of the dried products. After incorporating leucine into the HECi as described earlier, the mixture or pure HECi was frozen at -80°C in a vertical ultra-freezer Forma™ 900 Series (Thermo Fisher Scientific Inc., Waltham, MA, USA) and subsequently dried using a bench-top freeze-dryer (Labconco Corporation, Kansas City, MO, USA).

In vivo Evaluation of Gastroprotective Efficacy

Animals

Female Swiss albino mice (25–30 g; 7 weeks old) from the Central Animal House of the UFG were used throughout the experiments. Animals were randomly allocated in separate cages and maintained under controlled temperature (22 ± 2°C) and luminosity (12 h light/dark cycle) with pellet food and water *ad libitum*. The animals underwent seven days of acclimatization before beginning the experiments. Noteworthy, all the *in vivo* studies were previously approved by the Ethics Committee in Animal Experimentation of the UFG (Date of Approval November 8th, 2021; Protocol: 073/21), according to the international principles for research involving animals.

Indomethacin-induced Gastric Mucosal Lesions

The animals' gastric mucosa lesions were induced by indomethacin, according to the method previously published (Djahanguiri, 1969). Mice were fasted for 16h and distributed into four groups with seven animals each, namely: vehicle-treated group (2% Tween® 80 aqueous solution p.o., negative control); SDCi-treated groups (150 and 300 mg/kg p.o.); and ranitidine-treated group (50 mg/kg p.o., positive control). The SDCi was first solubilized in the vehicle at different concentrations depending on the body weight and the mice received 10 mL/kg of working solutions. Indomethacin was dissolved in $NaHCO_3$ solution (5%). All drugs and reagents were prepared immediately before use and dissolved in distilled water.

After sixty minutes, all groups received an indomethacin (50 mg/kg s.c) solution. Six hours after indomethacin administration, the animals were euthanized, and the stomachs were excised and opened along the greater curvature to examine the lesions. The parameters for determining the lesion index were the color of the mucosa, loss of mucosal folds and mucus, edema, petechiae, and the number of ulcers as previously described by Martins et al. (2014). The degree of ulceration was scored as follows: discoloration of mucosa, edema, or hemorrhages scored 1; number of petechia until 25% scored 2, while more than 25% scored 3. Scoring the intensity of ulceration considered the number of stomach lesions (N) x 2 if ulcers or erosion up to 1 mm, and N x 3 if larger than 1 mm.

Cytokine Levels Assay

Gastric tissue was scraped, weighed, and homogenized in heparinized phosphate buffer (pH 7.4) in a ratio of 1:5 and then centrifuged at 9000 x g for 20 minutes at 4º C. Aliquots of the supernatants obtained were used to estimate the levels of IL-1β and IL-10 using enzyme-linked immunosorbent assay (ELISA) kits purchased from Affymetrix eBioscience (San Diego, CA, USA),

according to the manufacturer's instructions. The plates were read at 450 nm using a microplate reader SpectraMax i3 (Molecular Devices, San José, CA, USA).

Statistical Analysis

For the *in vitro* experiments, data from three or more populations were compared using a one-way analysis of variance (ANOVA), followed by Tukey's test. Unpaired Student's t-test was used for pairwise comparisons. Mean comparisons besides regression and linear correlation analyses, were performed using GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA).

Regarding DoE, multivariate statistical analyses of the experimental data were conducted using RSM using RStudio (Posit Software, PBC, USA). The applied response function followed a quadratic polynomial equation, as defined in Eq. 2:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} {x_1}^2 + \beta_{22} {x_2}^2 + \beta_{33} {x_3}^2$$

2

In this equation, Y represents the predicted response (dependent variables); β_0 is the model constant or intercept; x_1, x_2, and x_3 are the independent variables; β_1, β_2, and β_3 are the linear coefficients; β_{12}, β_{13}, and β_{23} are the interaction coefficients; and β_{11}, β_{22}, and β_{33} are the quadratic coefficients. Response surface plots were also generated using RStudio.

For the *in vivo* experiments, data from three or more populations were compared using a one-way analysis of variance (ANOVA), followed by the Newman–Keuls test. Unpaired Student's t-test was used for pairwise comparisons. The GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA) was used. In all analyses, *p*-values less than 5% ($p < 0.05$) within a 95% confidence interval were considered statistically significant.

Results and Discussion

Standardization of Feeding Extract

Approximately 8 L of the extract was obtained. The EY was 44.9% (m/m), and the HECi had ρ of 1.05 ± 0,00 g/mL, pH 6.9, and Sc of 9.4 ± 0.2%. Moreover, the TPc and TFc were of 32.8 ± 1.5 mg/g, and 29.5 ± 0.6 mg/g, respectively. This TPc is comparable to that obtained for the ethyl acetate leaf extract of *C. iguanaea* (EAECi, 34.6 ± 0.3 mg/g gallic acid equivalent), which contained 15% of Sc. On the other hand, the TFc obtained for the HECi was, on average, 6-fold higher than EAECi (4.9 ± 0.1 mg/g, rutin equivalent) (Almeida et al., 2024). Unlike the Soxhlet extraction used for obtaining the EAECi, the percolation is an exhaustive process that does not involve heating,

It was impossible to determine the TPSc in the extract due to its low solubility in dichloromethane, which is used as the solvent in the Liebermann–Burchard method (Araújo et al., 2013). While the EY is high, the Sc is considered low, as a 30% concentration is recommended for feeding a spray dryer (Goula and Adamopoulos, 2004). This recommendation stems from the fact that more diluted solutions produce very fine dry particles that can be dragged by the air, reducing the drying yield (Filková and Mujumdar, 1995). However, this can be circumvented by using drying adjuvants (Couto et al., 2011; Sharapin et al., 2000). Furthermore, it is estimated that the heat consumption per kilogram of powder is 23,650 kJ for a feed with a solids content of 10%. This consumption drops to 6,170 kJ per kilogram of powder for a 30% solids content in the feed, resulting in a reduction of approximately 74% in heat consumption during the process (Filková and Mujumdar, 1995).

Concerning its reducing capacity, the IC_{50} was 1,429.7 ± 216.4 µg/mL. In light of its *in vitro* antioxidant capacity, the redox profile of the HECi, as determined by electrochemical techniques, is presented in Figure S1 of the Supplementary Material. The DPV revealed three anodic peaks (Fig. S1a). The first peak was reversible, with a potential of 0.26 V, a current of 3.80 µA, and an electrochemical index of 14.6 µA/V. Additionally, the two other anodic peaks, which were irreversible, occurred at potentials of 0.51 and 0.79 V, with currents of 0.49 and 0.81 µA and electrochemical indices of 0.95 and 1.03 µA/V, respectively. These findings confirm the antioxidant capacity of HECi, which has an overall electrochemical index of 16.6 µA/V. In comparison, the EAECi presented an electrochemical index of 42.7 µA/V and five anodic peaks in DPV at potentials of 0.015 V, 0.254 V, 0.370 V, 0.844 V, and 1.060 V. The SWV analysis of both HECi (Fig. S1b) and EAECi (Almeida et al., 2024) revealed two anodic peaks and one

cathodic peak, indicating the reversibility of redox pairs at 0.254 V due to the equivalence of kinetic parameters (Ipa/Ipc ≈ 1). Therefore, these analyses may contribute to assuring the identity and quality of *C. iguanaea* leaf derivatives.

Selection of Drying Aid

The SDCi obtained from the pilot test (SDCi0) showed a DY of 4.1%, TPc of 13.2 ± 0.5 mg/g, TFc of 29.1 ± 0.3 mg/g, and an IC_{50} of 638.0 ± 46.3 µg/mL. These results indicate an average loss of 59.8% in TPc (p-value < 0.05) and 1.4% in TFc (p-value > 0.05) compared to HECi. Notably, the reducing capacity of this SDCi was twice as high as that of HECi (p-value < 0.05), demonstrating that despite the significant reduction in polyphenol content, the *in vitro* reducing capacity of the extract remains preserved. As previously mentioned, the primary challenges associated with SDCi0 were its low recovery yield and high hygroscopicity. Many plant powders obtained by spray-drying aqueous, alcoholic, or hydroethanolic extracts exhibit some degree of hygroscopicity, usually attributed to the presence of significant amounts of hydrophilic compounds, such as carbohydrates, glycosides, organic acids, phenolics, amino acids, and proteins, dried at temperatures above the glass transition temperature (Tg) of the extract. This characteristic can cause concerns during the drying process and in subsequent formulation processes.

To address this issue, the inlet air temperature can be increased, or high molecular weight adjuvants can be added. Since increasing the temperature would raise the risk of degrading the extract's compounds of interest, using adjuvants was chosen as the preferred solution (Selvamuthukumaran, 2019). Figure S2 of Supplementary Material illustrates the characteristics of the spray-dried extracts obtained from the preliminary evaluation of different drying adjuvants. Leucine was selected due to its lower adherence to the plate and its known ability to decrease the hygroscopicity of spray-dried herbal extracts, thus corroborating previous findings (Chang et al., 2014).

Various drying adjuvants minimize adhesion issues to the drying chamber and raise powder recovery, including high molecular weight polymers such as maltodextrins, gums, and proteins. These compounds facilitate the drying process primarily by increasing the glass transition temperature (Tg). Additionally, the adjuvants can reduce surface tension, decreasing the material's adherence to other low-energy solid surfaces. This adhesion is the main cause of operational problems during drying and, consequently, leads to low yields and economic losses (Selvamuthukumaran, 2019). These excipients should be chemically inert, non-toxic, soluble, and thermally stable. Furthermore, drying adjuvants typically have high molecular weight, elevated Tg, and good surface-active properties, which produce low-viscosity solutions at high concentrations, resulting in denser particles. Additionally, adjuvants help prevent the loss of heat-sensitive components, such as phenolics, anthocyanins, vitamins, and carotenoids. The ideal proportions of these adjuvants must be determined for each specific process, taking into account both the composition of the extract and the drying conditions used (Selvamuthukumaran, 2019).

For use as intermediate products in the subsequent preparation of solid pharmaceutical forms, the drying adjuvant used for producing dried extract must be effective while minimizing the amount of excipient required. This approach helps reduce the number of tablets or capsules needed per dose (Chang et al., 2014). Leucine is widely used to modify the surface of spray-dried powders, resulting in particles with desirable properties. As a hydrophobic amino acid with a hydrophilic side chain, leucine possesses moderate surfactant properties. During spray drying, leucine molecules accumulate on the droplet surfaces, orienting their hydrophobic regions toward the air phase (Alhajj et al., 2021).

FDA classifies leucine as Generally Recognized as Safe (GRAS). It facilitates the maintenance of the shape and integrity of spray-dried particles, exhibiting anti-caking effects, preventing deliquescence at high relative humidities, and aiding in water desorption at low relative humidity (Chang et al., 2014). The Tg of leucine is reported as 4.85°C (Borredon et al., 2023), a low value for a drying adjuvant. Additionally, a study demonstrated that adding leucine in herbal extracts did not significantly increase the Tg compared to the corresponding powders without excipients. Thus, it is suggested that leucine behaves as a surfactant, tending to accumulate at air-solution interfaces, leading to its enrichment on the particle surface during spray drying. The leucine-rich surface reduces the active sites available for water absorption, preventing particle-particle bridge formation, subsequent agglomeration, and potential deliquescence (Chang et al., 2014).

Outcomes of the Spray Drying Process

Table 1 presents the attributes of spray drying quality under different experimental conditions. Compared to SDCi0, the addition of leucine significantly improved the DY. In experiments conducted under the same conditions as the pilot test (central point: IT of

120°C and Ef of 0.5 L/h), with the addition of 30% leucine relative to the solid content (SDCi 13, 14, and 15), the DY averaged 58%. Thus, the incorporation of 30% leucine resulted in a remarkable 14-fold increase in DY. The DY of the experiments conducted using the Box-Behnken design ranged from 22.67–62.02%, with an average of 46.52%. This was similar to the *Pistacia lentiscus* spray-dried powder (23.53% – 65.51%) (Jović et al., 2023). It is recommended that the yield of the product obtained on a laboratory scale should be at least 50% to achieve satisfactory spray drying efficiency at an industrial scale (Jović et al., 2023). In this regard, experiments 8, 10, 11, 12, 13, 14, and 15 surpassed this value, while conditions 1 and 4 were close to it, indicating that these conditions are feasible for application at an industrial scale.

The range of Mc of the SDCi (8.3–13.5%) obtained was comparable to the chamomile powder (7.5% – 12.0%) (S. Y. Lee et al., 2022), but higher than that of rosemary powder (< 5.0%) (Chaul et al., 2017), both obtained by spray-drying. A Mc lower than 5% is ideal for pharmaceutical powders (BRASIL, 2019). However, it is worth discussing that low Mc can impair the stability of the dried extract during subsequent storage. This is because powders with lower Lc may absorb more moisture from the environment due to the greater differences in water concentration between the powder and the storage environment. Since Mc and water activity are not linearly correlated (Juarez-Enriquez et al., 2019), the physicochemical, microbiological, and flow properties of the developing SDCi may not be negatively affected. Most of the SDCi obtained exhibited free-flowing properties and low hygroscopicity, which remained stable throughout the storage period. A short-term stability study is essential to assess the impact of storage conditions on the physicochemical quality attributes of the dried products, which is a key focus for further investigations by our research group.

Compared to SDCi0, the TPc and TFc of the products obtained under the same drying conditions and supplemented with 30% leucine (SDCi 13, 14, and 15) were significantly increased, averaging 17.37 mg/g and 36.56 mg/g, respectively. This represents an average enhancement of 32% (p-value < 0.05) and 26% (p-value < 0.05), respectively. The IC_{50} also increased, but no significant difference was observed compared with the extract without leucine. Therefore, it can be concluded that including leucine as a drying adjuvant improved the physicochemical properties of the dried extract. The TPc loss ranged from 29.7% (SDCi4) to 65.7% (SDCi9) compared with HECi (p-value < 0.05). The SDCi obtained at a lower IT (100°C) exhibited a TFc loss of up to 59.6% (SDCi7). Conversely, when intermediate or higher ITs (120°C and 140°C, respectively) were applied, spray drying improved the TFc, with results ranging from a 10.4% increase (SDCi9) to 68.4% (SDCi11). While the reducing capacity (IC_{50}) of the HECi was enhanced by up to 7.3-fold (SDCi10) following spray drying, its overall antioxidant capacity, as indicated by the EI, decreased by 33.4% (SDCi7) to 91.8% (SDCi6). Therefore, precise control of the operational conditions during spray drying is crucial to ensure the stability of phytochemicals in the product and, consequently, its biological activities.

The dried extracts obtained from the spray drying optimization showed a similar electrochemical profile. Like the HECi, they all exhibited an anodic peak (1a) in the range of 0.15 to 0.35 V, this time with a potential around 0.26 V. Anodic peaks below 0.5 V confirm the antioxidant activity of these extracts. Additionally, from the square wave voltammogram, it can be concluded that this is a reversible oxidation reaction, further reinforcing the high antioxidant power of the dried extracts. Except for SDCi 4, 6, and 12, the dried products exhibited a second peak around 0.74 V, and SCDi 2, 3, and 12 showed a peak at 0.83 V. However, these peaks do not have a significant influence on antioxidant activity, as they have anodic potentials greater than 0.5 V and low currents. The electrochemical behavior of the several SDCi are detailed in Table S1 and Figures S3-17 of the Supplementary Material.

The thermal efficiency (η) ranged from 28.75–49.63%, with an average of 35.81%. Spray dryers are the most energy-efficient drying systems, influenced by various factors, including drying chamber temperature, pressure, mass flow rate of the drying medium, feed mass flow rate, and air velocity (Filková and Mujumdar, 1995). Determining a suitable energy efficiency for bench-top spray dryers such as the one herein used can be challenging due to the limited specific data available for these smaller-scale units. However, insights from industrial-scale spray dryers can provide a general understanding. A survey conducted by the U.K. government's Energy Efficiency Best Practice Programme revealed that industrial spray dryers have specific energy consumptions ranging from approximately 3 to 20 GJ per ton of water evaporated, with an average of 4.87 GJ/t. This indicates that around 29% of the energy supplied to these dryers was wasted (Baker and McKenzie, 2005). In another study focusing on spray dryers with hybrid heat recovery systems, the highest exergy efficiency achieved was 49.9% at a dead state temperature of 10°C. In contrast, systems with direct heat recovery had lower efficiencies, around 19.17% at a drying air temperature of 500°C (Patel et al., 2022). Given the variability in designs and applications, it's essential to consult the manufacturer's specifications and conduct empirical

assessments to determine the energy efficiency of a specific bench-top spray dryer model. Implementing energy-saving measures, such as optimizing operating parameters and considering heat recovery options, can further improve efficiency.

The matrix showing the correlation between all the variables (both independent and dependent) involved in the Box-Behnken factorial design is displayed in Table 2. As expected, the main correlations were related to the temperatures: the IT showed a strong correlation with the Ti, which, in turn, had a strong correlation with the Tf, which also showed a strong correlation with the IT. Thermal efficiency was strongly positively correlated with the Ef. This behavior is likely since, at low Ef, less energy is required to dry the extract, resulting in higher outlet temperatures, which leads to a decrease in η. The TPSc had a strong correlation with the Lc, while the TPc showed a moderate to strong positive correlation with Lc. This suggests that the use of leucine was effective in protecting the extract during drying and maintaining the integrity of the bioactive compounds.

The TPc showed a strong negative correlation with the IC_{50}, such that an increase in TPc reduces this concentration, indicating an enhancement in the reducing capacity. Either TFc of TPSc did not significantly correlate with IC_{50}. The EI had a strong negative correlation with the IT during drying. This is likely because, at lower temperatures, antioxidant compounds are better preserved. Neither the contents of phytochemicals (TPc, TFc, and TPSc) nor the reducing capacity correlated with the EI. Therefore, the overall antioxidant capacity of the SDCis depends on the full spectrum of their phytochemicals rather than on specific classes, which reinforces the synergism between them. Furthermore, the reducing capacity of the extract is not the primary mechanism contributing to its *in vitro* antioxidant activity.

Influence of In-process Variables on Spray Drying

Given the multivariate nature of the DoE used, interpreting the observed effects is a complex task. Therefore, the data were subjected to RSM and correlation matrix analysis to clarify the relationships between in-process parameters and drying quality attributes. Table 3 summarizes the RSM results, presenting the adjusted (coded) coefficients obtained through Eq. 2 for each response variable, as well as for the overall optimization combining the significant parameters (OO). For clarification, the higher the coefficient value, the more significant the effect. Additionally, the table includes the coefficient of determination (R^2), F-statistic, *p*-value, and lack of fit. Bold values indicate statistical significance at different levels. The table also highlights the independent variables (in-process parameters) that optimize each response variable (quality attribute), along with their uncoded values at these optimal points (IT_{ot}, Ef_{ot}, and Lc_{ot}), and the predicted value of each response variable at the overall optimal point (Y_{ot}). Complementary, response surface plots provide a three-dimensional visual representation of the data to aid interpretation (Figs. 1–4).

The in-process parameters studied significantly impacted neither the Mc nor the TPc. Although Table 3 indicates that some linear or quadratic terms related to the studied factors significantly affected TPSc (Lc), IC_{50} (Ef^2), EI (IT), and η (Ef), the F-statistics indicate that the model's predictive capacity (indicated by the R^2) for these outcomes was not substantial, as the *p*-values were above 0.05.

The DY was significantly and positively influenced by the linear coefficients of the Ef and Lc, and by the quadratic coefficients of the IT (IT^2) and Lc IT (Lc^2), both with negative effects. As depicted in Fig. 1, as the IT increases, the DY rises to approximately 120ºC and then decreases (Figs. 1a and 1b). At lower IT, the drying process is insufficient, leading to slower droplet drying, which causes agglomeration and deposition on the drying chamber walls, ultimately reducing the yield. At higher temperatures, the droplets dry too quickly, producing smaller particles that are carried away by the drying air toward the overflow, resulting in lower yields as well. Therefore, the DY tends to be higher at intermediate temperature values. Notably, the yield values were considerably lower at the highest temperature within the experimental range, indicating that this particle carryover loss is more detrimental to the process under investigation. Regarding the Ef, although the quadratic and interaction terms were insignificant, the response surface showed curvature, which seems to interact with IT. When IT is fixed, the linear influence becomes more evident, with DY increasing as the feed flow rate rises (Figs. 1a and 1c). This occurs because a higher Ef enhances mass transfer, leading to more effective droplet drying and improved yield.

The DY increases with the Lc up to approximately 30%, after which it begins to decrease, forming a parabolic relationship between the two variables when the Ef is fixed (Fig. 1b). However, when the IT is fixed, the yield is low and gradually increases with the Lc at lower Ef. At higher Ef, the yield also increases with Lc but only up to a certain point, beyond which it starts to decrease (Fig. 1c).

At low Ef, increasing the Lc raises the solids content in the droplet, consequently reducing the water proportion, which facilitates drying and enhances yield. At high Ef, this effect also occurs but only to a certain extent. Beyond this point, the increased leucine content makes the droplets too heavy, causing them to settle in the drying chamber, reducing yield (Dao et al., 2021). Moreover, the excessive addition of drying adjuvants can increase the feed fluid's viscosity, leading to the atomization of larger droplets and hindering mass transfer from the droplet's interior to its surface. In such cases, longer drying times are required, resulting in less efficient recovery and powders with lower secondary metabolite contents (Dao et al., 2021). The response surface for DY exhibited maxima in all response surface graphs. This optimal point occurs at IT of 122.62°C, Ef of 0.58 L/h, and Lc of 36.08%, yielding 60.48%. Notably, this is also the point optimized by the mathematical model. Figure S18 of the Supplementary Material details these optimal points.

The TFc was significantly influenced by the quadratic and linear components of the IT, the interaction between IT and Lc, and the linear component of the Ef. The TFc increases with the rise in IT up to a certain point, after which it starts to decrease (Figs. 2a and 2b). The initial increase in flavonoid content is likely due to the enhanced drying efficiency at higher temperatures. However, at elevated temperatures, thermal degradation of these compounds occurs, leading to a reduction in TFc. The interaction between Lc and IT was also significant (Fig. 2b). At lower IT, increasing the amount of adjuvant reduces TFc, while at higher IT, increasing Lc e enhances TFc. When the IT is fixed (Fig. 2c), raising Lc leads to a sharp linear increase in TFc at lower Ef. In contrast, at higher Ef, TFc slightly decreases with increasing Lc.

Although increasing Lc improves drying efficiency by raising the solid content, it also makes the particles heavier and slower, thus increasing their residence time inside the equipment. Consequently, prolonged exposure to hot air results in the loss of thermolabile compounds. This effect is more pronounced at lower IT, whereas at higher ITs, the faster drying rate minimizes this impact. Additionally, at elevated IT, the increased Lc enhances flavonoid retention due to the thermal protection offered by the leucine coating on the particle surface. In the optimization study on firethorn (*Pyracantha coccinea*), the most significant variable was the adjuvant proportion, which effectively protected phenolic compounds by acting as a wall-forming agent (Dincer and Temiz, 2023).

The Ef also linearly and significantly influences TFc (Figs. 2a and 2c). As the Ef increases, TFc decreases. Although interactions involving Ef are not statistically significant, they noticeably affect the response surface. When Lc is fixed (Fig. 2a), TFc remains relatively constant at low IT but sharply decreases with increasing Ef at higher IT. Conversely, when the IT is fixed (Fig. 2c), TFc sharply increases with decreasing Ef at higher Lc. At lower Lc, this influence is less pronounced, following the same trend but with a smaller variation. Generally, increasing the Ef should enhance flavonoid retention by shortening the particle's residence time in the equipment, thereby reducing compound degradation due to high temperatures. At lower temperatures, increasing the Ef has a less significant impact.

As expected, the linear coefficient of the IT was statistically significant for both Ti and Tf, predominantly influencing the responses (Figs. 3a and 3b). As a result, the outlet temperatures increase with the rise in the IT during the drying process. The Ef was also significant for both the Ti and Tf, although its influence was minimal, with an increase in Ef leading to a decrease in either Ti or Tf (Figs. 3a and 3c). The Lc, in turn, seems to interact without statistical significance with the Ef (Fig. 3c). Due to this interaction, an increase in Lc raises the outlet temperatures at low Ef, while at high Ef, an increase in Lc results in a decrease in these temperatures.

Optimization of Spray Drying

By optimizing the variables that showed significant multivariate regressions, the response surface for the overall drying optimization can be represented by **Eq. 3**:

$$OO = DY + TFc \quad \textbf{(3)}$$

The general equation for the spray drying process optimization was significant and showed no significant lack of fit (Table 3), indicating that the model adequately represents the experimental data. The Lc, IT^2, and the interaction between Lc and IT were significant. The IT has a quadratic effect on the drying process but interacts with the Lc (Figs. 4a and 4b). Thus, at high levels of adjuvant, increasing the IT to higher levels does not negatively impact the process as much as it does at lower Lc. This occurs because, at lower Lc, increasing the IT enhances the process by improving heat and mass transfer and boosting thermal

efficiency. However, beyond a certain point, further IT increases impair the drying process by degrading bioactive compounds and reducing DY due to the loss of lighter (over-dried) particles through the overflow. At high Lc, this negative effect is less pronounced, as the increased Lc leads to the formation of heavier particles with a coating that protects thermolabile compounds.

The Ef did not show significant components, but from the graphical analysis, it behaved quadratically, improving the process at intermediate flow rates and impairing it at extreme values (Figs. 4a and 4c). However, this effect was more pronounced at higher temperatures. When the temperature was fixed (Fig. 4c), the process improved at low Lc with increasing Ef, but at high Lcs, increasing the Ef hindered the drying process. At low temperatures, the process was better at intermediate leucine levels, although this improvement was minimal. In contrast, at high temperatures, increasing Lc consistently enhanced the process. This occurs because higher leucine levels facilitate drying by increasing the solids content, thereby improving mass transfer, and protecting bioactive compounds from thermal degradation. Leucine addition was crucial for the spray drying of *C. iguanaea*, as statistically, increasing the adjuvant amount always improved the drying process. It is worth noting that this improvement was more significant at higher temperatures and lower Ef.

Thus, optimal drying occurred at medium to high IT, low Ef, and high Lc. The optimal point was at IT of 130°C, an Ef of 0.3 L/h, and 45% (w/w) leucine relative to the dry residue of the feed extract. This condition enabled a good yield of 52.55% and satisfactory levels of total phenolics, flavonoids, and phytosterols. However, the energy efficiency of 28.96% was considered low, primarily due to the low Ef. The process attributes at this optimal point indicate rapid drying but with high energy requirements. Starting with the raw material, the solids content was low, which increased energy demand. For extracts with 10% solids content, a heat consumption of 23,650 kJ per kg of dry powder is expected (Filková and Mujumdar, 1995). Therefore, further studies should be conducted to reduce this energy demand. Among the factorial design experiments, SDCi11 (IT of 120°C, Ef of 0.3 L/h, and Lc of 45% m/m) was selected as the optimized SDCi because it was the closest to the optimal point.

Morphology of the Optimized Spray-dried Extract

The SDCi11 underwent SEM to evaluate the morphology of its particles. Figure 5 displays the particle diameter measurements and their corresponding histogram, and Fig. 6 presents its microscopic images at different magnifications. A total of 244 observations were made, ranging from 0.7 to 13.1 μm, with an average of 3.4 ± 2.1 μm. Therefore, it can be stated that microparticles were produced. These values align with the expected particle size range (5 to 50 μm) for pharmaceutical products obtained through spray drying (Filková and Mujumdar, 1995). The particles appeared to be hollow and corrugated (as seen in Fig. 6b), which was anticipated due to the use of leucine. However, dense corrugated particles were also observed, as shown in Fig. 6c. This wrinkled, concave outer surface is characteristic of all spray-dried powders (Dao et al., 2021). Additionally, some agglomerates of smaller particles were observed.

Particle size and morphology are influenced by feed material-related factors such as solid content, viscosity, surface tension, flow rate, and type of drying aid. A feed with low solid content decreases drying efficiency, requiring more energy to remove a greater solvent volume. Smaller particles are also produced, which increases the amount of material carried by the drying air to the overflow aid (Walton, 2000; Walton and Mumford, 1999). To address this issue, drying aids are recommended to promote the formation of larger particles and optimize yield (Sharapin et al., 2000; Ziaee et al., 2019). Another consequence of low solid content in the feed is the reduction in wall thickness, which increases the hollow internal space of the particles. Other important factors to consider are the viscosity and surface tension of the feed, as they influence the formation of spherical droplets (Walton, 2000; Walton and Mumford, 1999). High viscosity hinders droplet formation during atomization, leading to larger particle sizes. Using surfactant substances, such as leucine, helps mitigate this effect by promoting the formation of smaller droplets and increasing atomization speed (Oliveira and Petrovick, 2010).

During spray drying from aqueous solutions such as the HECi, leucine's low water solubility leads to its rapid saturation at the droplet surface, causing crystallization that forms a hydrophobic outer shell around hollow particles. As solvent evaporation continues, this outer layer collapses, resulting in wrinkled particles. The hydrophobic nature of the external shell reduces interparticle forces, enhancing the powder's flowability and dispersibility. The influence of leucine on the morphology of a dry particle depends on its ability to coat the surface of the dried droplets. Therefore, factors such as the proportion of leucine, the nature of the solvent system (aqueous, organic, or a mixture of both), the pH of the solvent system, and the processing

temperature will dictate its effects on particle morphology. Systems with low Lc result in wrinkled particles with minimal leucine surface coverage and subtle morphological differences. Increasing the Lc enhances the molar percentage of leucine on the surface and the degree of corrugation, but only up to a certain limit. The presence of ethanol leads to the formation of hollow particles. This occurs because leucine precipitates rapidly on the droplet surface due to its lower solubility in ethanol, forming a shell around a hollow particle. Processing temperature significantly influences the distribution of leucine on drying droplets, thereby affecting the morphology of the resulting particles. Lower inlet temperatures tend to produce spherical particles with smoother surfaces. Conversely, the evaporation rate is increased by higher temperatures, leading to smoother particle surfaces as well (Alhajj et al., 2021).

Identification of Major Compounds in the Optimized SDCi

The results presented in Figure S19 and Table 4 indicate the presence of a hydroxycinnamic acid (peak 2) and two flavones (peaks 7 and 8) as the major compounds in the SDCi11. A literature review was conducted to identify potential substances corresponding to these major compounds based on the chromatograms obtained from LC-PDA, LC-UV, and ESI(-)-MS analyses. The suggested phytochemicals are listed in Table 4, along with the references that support these propositions.

p-Coumaric acid has previously been reported as a constituent of *C. iguanaea* (Almeida et al., 2024). Herein, a *p*-coumaric acid glycoside was identified, consisting of *p*-coumaric acid linked to a sugar moiety. *p*-Coumaric acid and its derivatives have been reported to exhibit antioxidant, antimicrobial, anticancer, antiarthritic, anti-inflammatory, antidiabetic, antimelanogenic, gastroprotective, antiulcer, cardioprotective, hepatoprotective, nephroprotective, antiangiogenic, and antiplatelet effects, in addition to preventing gout and promoting skin regeneration and bone formation (Kaur and Kaur, 2022).

Luteolin-*C*-hexoside-*O*-pentoside is a flavonoid glycoside derived from luteolin, a flavone. This compound had not been previously reported for this species. However, other luteolin derivatives have been mentioned, including luteolin-4'-*O*-rhamnosyl (1→2) glycoside and luteolin-7-*O*-glucoside (Almeida et al., 2024). No pharmacological data on luteolin-*C*-hexoside-*O*-pentoside were found in the scientific literature. However, luteolin is known for its antioxidant, anticancer, anti-inflammatory, antibacterial, antifungal, and antiviral activities. In addition, luteolin helps regulate skin aging and inflammation, mitigates fibrosis and liver inflammation, protects the kidneys, and has antidiabetic and antihypertensive properties. Its neuroprotective effects have also been reported, indicating that luteolin inhibits oxidative stress, neuroinflammation, and neuroinflammatory responses, protecting against neuroinflammatory diseases such as Parkinson's disease, Alzheimer's disease, and Multiple Sclerosis (Bangar et al., 2023; Kahrizi and Mohammadi, 2023).

Despite the broad range of therapeutic potentials of luteolin, there remains a scarcity of information regarding its bioavailability, absorption, and metabolism—essential aspects for understanding its therapeutic effects and safety profile, particularly for its derivatives (Bangar et al., 2023). Additionally, it has been demonstrated that the reactivities of luteolin and some of its glycoside derivatives are dependent on their molecular structures. The presence of ortho-dihydroxy groups on the B ring and the OH substitution pattern at the C-5 position on the A ring can significantly contribute to its anti-inflammatory and antioxidant effects (Odontuya et al., 2005).

The presence of 2-*O*-pentosyl-8-*C*-hexosyl-apigenin, another flavone, was also previously reported in other *C. iguanaea* leaf extracts (Almeida et al., 2024; Zanchet et al., 2018), suggesting that this compound could be a phytochemical marker for the species. Identifying a phytochemical marker for *C. iguanaea* would enable the development and validation of an HPLC-PDA method for quantification in quality control and pharmacokinetics studies. However, to the best of our knowledge, no pure standard is currently available on the market. Therefore, isolating and purifying considerable amounts of this phytochemical are among the primary objectives of our research team's future work.

No pharmacological data was found in the literature for this compound; however, apigenin exhibits anti-inflammatory, anticancer, antidiabetic, anti-obesity, antidepressant, and hepatoprotective effects. It also shows promise in treating insomnia, infections, and respiratory, cardiovascular, neurodegenerative, and skin diseases (Mushtaq et al., 2023). Flavones are an important class of bioactive compounds known for their anti-inflammatory activity, and their structural diversity allows interaction with different targets. It has been reported that subtle structural variations in flavones, due to the position and number of hydroxyl groups and the degree of methoxylation, are associated with significant variations in the in vitro anti-inflammatory activity of flavones.

Hydroxyl groups are known to be essential for this activity; however, hydroxyls at the C-5 and C-4' positions enhance the effect, whereas those at the C-6, C-7, C-8, and C-3' positions attenuate it (Wang et al., 2021). Therefore, further studies are needed to investigate the bioactivity of the specific compounds reported in this study for *C. iguanaea*.

Spray Drying vs. Freeze-drying

The TPc, TFc, and TPSc for the extracts freeze-dried without adding leucine were 4.32 ± 0.02 mg/g, 17.42 ± 0.16 mg/g, and 39.97 ± 1.57 mg/g, respectively; while for the extract with 45% leucine added, they were 14.55 ± 0.53 mg/g, 58.99 ± 0.33 mg/g, and 51.77 ± 2.64 mg/g, respectively. The levels of phenolics and flavonoids increased significantly (p-value < 0.05), tripling the content in the freeze-dried extract with leucine compared to that without leucine. Therefore, adding leucine significantly improved the content of these phytochemicals in the freeze-dried extract. The phytosterol content, however, was not significantly different (p-value > 0.05).

When comparing the results of the freeze-dried and spray-dried extracts in the absence of leucine (SDCi0), the TPc was 3.4-fold higher (p-value < 0.05) in the spray-dried extract compared to the freeze-dried, while the TFc was 1.6-fold higher (p-value < 0.05). Therefore, it can be stated that the preservation of phenolics and flavonoids was enhanced in the spray drying, despite using high temperatures, likely due to the shorter residence time within the drying chamber. These findings corroborate with other previously obtained when comparing freeze- and spray drying of papaya pulp (Gomes et al., 2018), protein-spinach particles (Grace et al., 2022), and olive leaf aqueous extract (Ghelichkhani et al., 2019). When comparing the results of the freeze-dried and spray-dried extract with 45% leucine (SDCi 7, 8, 11, and 12 from Table 1), it was observed that the TPc and TPSc were significantly higher (p-value < 0.05) in the spray-dried extract, while the TFc was lower (p-value < 0.05).

Contrary to common belief, freeze-drying cannot always be considered more appropriate than spray-drying for heat-sensitive compounds. Both methods are effective at preserving thermolabile substances. This efficiency seems to depend on the raw material and excipients used, as well as the desired product characteristics. An extensive review of the advantages and limitations of each technique is presented elsewhere (Buljeta et al., 2022; Kandasamy and Naveen, 2022).

SDCi Reduces Indomethacin-induced Gastric Mucosal Lesion In Vivo, Likely Owing to Its Antioxidant and Immunomodulatory Properties

Based on the data in Table 1, animals treated with SDCi11 at 150 mg/kg received 3.3 mg/kg of polyphenols, 7.5 mg/kg of flavonoids, and 77.8 mg/kg of phytosterols, while those treated with 300 mg/kg received 6.6 mg/kg, 15.0 mg/kg, and 155.6 mg/kg, respectively. Figure 7 shows that oral administration (p.o.) of SDCi11 at doses of 150 mg/kg and 300 mg/kg significantly reduced mucosal lesions by 41% (12 ± 1.95) and 45% (11.25 ± 0.85), respectively, compared to the vehicle group (20.33 ± 1.9). Moreover, the positive control, ranitidine, demonstrated a lesion reduction rate of 65% (7 ± 0.97). This decrease observed in the group treated with SDCi11 at 300 mg/kg was comparable to our previous findings with the crude *C. iguanaea* ethanolic leaf extract at the same dose (De Sousa et al., 2013), indicating that the spray-drying process preserved the bioactive compounds responsible for these protective effects. Although the lesion reduction rate obtained for SDCi11 was lower than that of ranitidine, the natural composition of the spray-dried extract offers an advantage in terms of safety and reduced side effects commonly associated with this synthetic drug, *e.g.*, headaches, tiredness, dizziness, diarrhea, constipation, and nausea (Shukla and Kher, 2021).

Indomethacin is a NSAID indicated for managing headaches (Summ et al., 2021), gouty arthritis pain, and other painful conditions (Nalamachu and Wortmann, 2014). The indiscriminate or excessive use of non-selective COX inhibitors has been associated with the incidence of peptic ulcers among other gastrointestinal side effects (Lucas, 2016). Indomethacin is suggested to induce gastric damage by inhibiting the release of protective factors such as COX-1, prostaglandin E2 (PGE2), bicarbonate, and mucus, while simultaneously increasing aggressive factors like gastric acid. Additionally, it elevates oxidative stress by increasing pro-oxidant parameters and decreasing antioxidant defenses (Suleyman et al., 2010).

Polyphenol- and flavonoid-rich herbal extracts, known for their potent antioxidant properties, counteract reactive oxygen species (ROS) involved in indomethacin-induced gastric damage by preventing ROS-mediated mitochondrial dysfunction and gastric mucosal apoptosis (Shukla and Kher, 2021). Additionally, flavonoids are reported to enhance the synthesis of protective gastric

mucus and modulate inflammatory pathways by inhibiting pro-inflammatory cytokines, such as TNF-α and IL-1β, while promoting anti-inflammatory cytokines like IL-10 (Serafim et al., 2020). Linoleic acid, or 9,12-octadecadienoic acid, a polyunsaturated fatty acid found in apolar *C. iguanaea* leaf extract with gastroprotective efficacy (Martins et al., 2014a), also contributes to the immunomodulatory effect by inhibiting inflammation (Martins et al., 2022).

As shown in Fig. 8a, oral treatment with SDCi11 at 150 mg/kg reduced IL-1β levels by 42.4% (45.6 ± 8.3) compared to the vehicle-treated group (79.1 ± 5.8). Additionally, Fig. 8b shows that oral administration of SDCi11 at doses of 150 and 300 mg/kg markedly increased IL-10 levels by 345% (1153 ± 278.7) and 390% (1272 ± 386.6), respectively, compared to the vehicle-treated group (259.1 ± 153.1). The observed modulation of cytokine levels by SDCi11 corroborates previous findings on the in vivo anti-inflammatory efficacy of *C. iguanaea* leaf extract (Almeida et al., 2024), while also aligning with results from other studies investigating the protective effects of natural compounds against indomethacin-induced gastric damage. For instance, carnosic acid has been shown to attenuate inflammatory damage by suppressing IL-1β production and enhancing antioxidant defenses, thereby exhibiting a gastroprotective effect (Danisman et al., 2023). Likewise, ellagic acid facilitates gastric ulcer healing by downregulating pro-inflammatory cytokines, including IL-1β, and upregulating anti-inflammatory mediators (Chatterjee et al., 2012).

The significant increase in IL-10 levels observed with SDCi11 treatment is particularly noteworthy, as IL-10 plays a fundamental role in maintaining mucosal homeostasis and modulating immune responses. Enhancing IL-10 levels can suppress undesired immune reactions and promote a non-inflammatory state, which is crucial for tissue repair and protection against ulceration (Nguyen et al., 2021). These findings suggest that the gastroprotective effects of SDCi11 may be mediated through its antioxidant activity and ability to modulate the immune response, reducing pro-inflammatory mediators while enhancing anti-inflammatory pathways. This dual action mitigates the direct tissue damage caused by indomethacin and promotes an environment conducive to mucosal healing. Noteworthy, by combining antioxidant, gastroprotective, antinociceptive, and anti-inflammatory efficacy *in vivo*, *C. iguanaea* leaf extracts emerge as a promising multi-target phytopharmaceutical alternative for managing illnesses related to oxidative stress.

Conclusion

The application of QbD methodologies, specifically the Box-Behnken DoE and RSM, has effectively optimized the spray drying of *Celtis iguanaea* hydroethanolic leaf extract. This optimization has led to satisfactory laboratory-scale powder recovery while preserving the extract's phytochemical integrity and antioxidant properties. Incorporating leucine as a drying aid significantly impacted the quality attributes of both spray-dried and freeze-dried powders. Notably, the optimized spray-dried extract demonstrated significant gastroprotective properties *in vivo* by modulation of IL-1β and IL-10 levels, highlighting its therapeutic potential in managing NSAID-induced gastric mucosal injuries. Further studies are warranted to enhance the system's energy efficiency and elucidate the precise mechanisms underlying these gastroprotective effects, aiming to explore the clinical applicability of this high-value intermediate phytopharmaceutical product in preventing and treating PUD.

Declarations

Competing Interests

The authors declare no conflict of interest. We certify that we have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Author Contributions

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Tables

Table 1: Box-Behnken factorial design matrix (3^3) with spray-drying process variables and quality attributes

SDCi	IT (°C)	Ef (L/h)	Lc (% m/m)	DY (% m/m)	Mc (% m/m)	TPc (mg/g)	TFc (mg/g)	TPSc (mg/g)	IC_{50} (µg/mL)	EI (µA/V)	Ti (°C)	Tf (°C)	η (%)
12	120 (0)	0.7 (+1)	45 (+1)	54.86	11.08±1.18	21.56±0.61	31.54±2.79	413.35±6.20	244.05±108.34	5.80	73.1	73.5	49.63
7	100 (-1)	0.5 (0)	45 (+1)	45.47	9.80±3.05	20.02±0.47	11.91±1.05	455.10±24.47	678.82±192.05	9.72	70.8	75.5	36.23
3	100 (-1)	0.7 (+1)	30 (0)	42.69	11.53±0.50	17.05±0.39	12.07±0.00	431.77±11.18	488.73±117.42	9.66	69.1	77.2	36.23
1	100 (-1)	0.3 (-1)	30 (0)	49.30	12.97±0.12	16.46±0.66	15.71±0.75	481.83±18.56	382.39±73.24	7.62	74.6	79.0	31.31
14	120 (0)	0.5 (0)	30 (0)	62.02	10.2±0.97	17.83±0.65	38.63±1.65	399.76±9.00	490.58±34.83	7.41	86.3	91.0	33.32
10	120 (0)	0.7 (+1)	15 (-1)	54.06	13.53±0.51	17.81±0.50	33.06±0.39	383.64±12.40	195.57±44.23	6.30	82.0	81.9	40.44
13	120 (0)	0.5 (0)	30 (0)	52.45	11.98±0.31	18.28±0.68	36.83±0.74	423.39±4.59	569.80±84.61	9.39	84.0	89.8	35.18
11	120 (0)	0.3 (-1)	45 (+1)	51.90	9.85±0.34	21.78±0.82	49.69±0.57	518.81±18.02	301.62±38.77	6.13	89.9	96.0	28.75
9	120 (0)	0.3 (-1)	15 (-1)	25.67	12.65±0.18	11.25±0.56	32.57±0.53	382.13±20.45	970.83±171.41	5.46	87.8	90.1	33.00
2	140 (+1)	0.3 (-1)	30 (0)	34.83	10.59±0.38	20.86±0.80	49.28±1.85	406.38±11.95	471.88±96.86	5.69	103.8	107.8	29.97
8	140 (+1)	0.5 (0)	45 (+1)	53.40	9.16±0.16	16.20±0.59	40.48±1.71	491.96±21.62	786.63±62.75	6.32	98.9	101.6	34.84
5	100 (-1)	0.5 (0)	15 (-1)	39.38	10.53±0.21	14.85±0.43	31.01±1.47	346.58±34.24	919.84±100.33	8.08	69.3	71.8	39.74
4	140 (+1)	0.7 (+1)	30 (0)	49.64	9.42±0.07	23.05±0.77	18.24±0.50	439.75±11.80	225.14±85.99	6.92	99.4	96.0	37.07
6	140 (+1)	0.5 (0)	15 (-1)	22.67	10.19±0.77	17.04±0.54	15.00±0.96	344.12±17.34	679.64±12.00	1.19	98.0	100.8	35.58
15	120 (0)	0.5 (0)	30 (0)	59.48	8.32±0.21	16.01±0.86	34.21±1.31	367.07±16.19	769.89±47.15	8.24	81.8	90.6	35.92

Spray-dried hydroethanolic leaf extract (SDCi); The studied in-process variables (independent) were drying air inlet temperature (IT,); extract feed flow rate (Ef), and leucine content (Lc). The process quality attributes (dependent variables) were drying yield (DY), moisture content (Mc), total phenolic content (TPc), total flavonoid content (TFc), total phytosterol content (TPSc), half-maximal inhibitory concentration (IC_{50}), electrochemical index (EI), initial outlet temperature (Ti), final outlet temperature (Tf), energy efficiency (η).

Table 2: Correlation matrix of the optimization parameters for the spray drying of *Celtis iguanaea* hydroethanolic leaf extract

	IT	Ef	Lc	DY	Mc	TPc	TFc	TPSc	IC_{50}	EI	Ti	Tf	η
IT	1.00	0.00	0.00	-0.13	-0.35	0.27	0.39	-0.06	-0.12	-0.66	0.94	0.87	-0.12
Ef	0.00	1.00	0.00	0.32	-0.03	0.28	-0.39	-0.22	-0.37	0.17	-0.26	-0.37	0.76
Lc	0.00	0.00	1.00	0.52	-0.45	0.57	0.16	0.77	-0.29	0.31	-0.04	0.02	0.01
DY	-0.13	0.32	0.52	1.00	-0.19	0.35	0.26	0.37	-0.44	0.52	-0.22	-0.19	0.20
Mc	-0.35	-0.03	-0.45	-0.19	1.00	-0.31	-0.13	-0.08	-0.22	0.01	-0.31	-0.37	0.08
TPc	0.27	0.28	0.57	0.35	-0.31	1.00	0.08	0.41	-0.76	0.03	0.21	0.15	0.13
TFc	0.39	-0.39	0.16	0.26	-0.13	0.08	1.00	0.10	-0.01	-0.13	0.43	0.47	-0.25
TPSc	-0.06	-0.22	0.77	0.37	-0.08	0.41	0.10	1.00	-0.37	0.28	0.07	0.10	-0.36
IC_{50}	-0.12	-0.37	-0.29	-0.44	-0.22	-0.76	-0.01	-0.37	1.00	0.02	-0.06	0.03	-0.18
EI	-0.66	0.17	0.31	0.52	0.01	0.03	-0.13	0.28	0.02	1.00	-0.61	-0.52	0.01
Ti	0.94	-0.26	-0.04	-0.22	-0.31	0.21	0.43	0.07	-0.06	-0.61	1.00	0.96	-0.44
Tf	0.87	-0.37	0.02	-0.19	-0.37	0.15	0.47	0.10	0.03	-0.52	0.96	1.00	-0.58
η	-0.12	0.76	0.01	0.20	0.08	0.13	-0.25	-0.36	-0.18	0.01	-0.44	-0.58	1.00

r = Pearson correlation coefficient. |0.0| < r ≤ |0.1| = no correlation; |0.1| < r ≤ |0.3| = weak correlation; |0.3| < r ≤ |0.6| = moderate correlation; |0.6| < r ≤ |0.9| = strong correlation; |0.9| < r < |1.0| = very strong correlation; r = 1.0 = perfect correlation (Callegari-Jacques, 2009); drying air inlet temperature (IT, ºC), extract feed flow rate (Ef, L/h), and leucine content (Lc, % m/m); drying yield (DY, % m/m), moisture content (Mc, % m/m), total phenolic content (TPc, mg/g), total flavonoid content (TFc, mg/g), total phytosterol content (TPSc, mg/g), half-maximal inhibitory concentration (IC_{50}, µg/mL), electrochemical index (EI, µA/V), initial outlet temperature (Ti, ºC), final outlet temperature (Tf, ºC), and energy efficiency (η, %).

Table 3
Summary of the RSM used for evaluating the effects of spray-drying process variables on the properties of *Celtis iguanaea* dried extracts

Paramters	DY (% m/m)	Mc (% m/m)	TPc (mg/g)	TFc (mg/g)	TPSc (mg/g)	IC_{50} (µg/mL)	EI (µA/V)	Ti (ºC)	Tf (ºC)	η (%)	OO
Intercept	**0.795[b]**	-0.282	0.037	0.305	-0.398	0.069	**0.679[a]**	-0.139	0.037	-0.420	**1.100[b]**
IT	-0.104	-0.263	0.186	**0.346[a]**	-0.047	-0.099	**-0.438[a]**	**0.838[c]**	**0.713[c]**	-0.072	0.242
Ef	**0.251[a]**	-0.025	0.193	**-0.346[a]**	-0.173	-0.314	0.111	**-0.234[a]**	**-0.308[b]**	**0.483[b]**	-0.095
Lc	**0.406[b]**	-0.336	0.394	0.145	**0.605[b]**	-0.243	0.203	-0.032	0.014	0.008	**0.551[a]**
IT x Ef	0.272	0.027	0.068	-0.362	0.239	-0.228	-0.047	0.016	-0.139	0.052	-0.090
IT x Lc	0.313	-0.029	-0.255	**0.590[b]**	0.113	0.224	0.205	-0.009	-0.040	0.066	**0.903[b]**
Ef x Lc	-0.323	0.033	-0.287	-0.247	-0.306	0.463	-0.069	-0.159	-0.199	0.322	-0.570
IT^2	**-0.515[a]**	-0.176	0.077	**-0.658[b]**	0.161	0.155	-0.055	0.108	0.044	-0.120	**-1.173[b]**
Ef^2	-0.190	0.536	0.259	-0.016	0.333	**-0.718[c]**	-0.150	0.047	-0.070	0.009	-0.206
Lc^2	**-0.387[a]**	0.073	-0.135	0.025	-0.016	0.248	-0.418	-0.095	-0.213	0.292	-0.363
R^2	**0.931**	0.595	0.735	**0.934**	0.872	0.851	0.808	**0.978**	**0.971**	0.855	**0.929**
F	7.535	0.815	1.543	7.806	3.773	3.169	2.340	25.040	18.780	3.285	7.230
p - value	**0.019[a]**	0.629	0.330	**0.018[a]**	0.079	0.109	0.181	**0.001[b]**	**0.002[b]**	0.102	**0.021[a]**
Lack of fit*	0.515	0.682	0.121	0.098	0.451	0.434	0.231	0.341	**0.022[a]**	0.105	0.249
IT_{ot}	122.62	140	140	139.74	100	140	100	100	100	124	130.54
Ef_{ot}	0.58	0.49	0.70	0.30	0.30	0.70	0.61	0.70	0.70	0.70	0.30
Lc_{ot}	36.08	45	21.80	45	45	16.56	29.32	45	45	45	45
Y_{ot}	60.48	8.28	22.23	62.77	548.38	66.73	10.16	63.05	67.69	46.49	135.85
Y_{OO}	52.55	10.31	20.69	60.14	515.07	364.77	6.12	97.42	102.66	28.96	135.85

Response Surface Methodology (RSM); The studied in-process variables (independent) were drying air inlet temperature (IT, ºC); extract feed flow rate (Ef, L/h), and leucine content (Lc, % m/m). The process quality attributes (dependent variables) were drying yield (DY), moisture content (Mc), total phenolic content (TPc), total flavonoid content (TFc), total phytosterol content (TPSc), half-maximal inhibitory concentration (IC_{50}), electrochemical index (EI), initial outlet temperature (Ti), final outlet temperature (Tf), energy efficiency (η), and overall optimization (OO); R^2 is the determination coefficient; **p*-value; Y_{ot} is the optimize predicted response for each quality attribute; Y_{GO} is the predicted response for each quality attribute in the global optimization of the system. Statistically significant at $^a p < 0.05$, $^b p < 0.01$, and $^c p < 0.001$ at the confidence interval of 95%.

Table 4
Major phytochemicals in the optimized spray-dried *C. iguanaea* extract (SDCi11) identified by LC-PDA and ESI(-)-MS

Peak	Tentative identification	Rt (min)	$\lambda_{máx.}$ (nm)	$[M-H]^-$	Molecular formula	MS/MS Literature	Method used	Species	Reference
2	*p*-coumaric acid glycoside	22.509	204, 280	325	$C_{15}H_{18}O_8$	163, 119	HPLC-DAD–MS/MS	*Rhinacanthus nasutus* (L.) kurz	(Huang et al., 2015)
7	luteolin-*C*-hexoside-*O*-pentoside	35.936	205, 268, 344	579	$C_{26}H_{28}O_{15}$	447 (15), 417 (7), 327 (7), 285 (100)	HPLC/DAD/ESI-MS-MS	*Chenopodium ambrosioides* L.	(Barros et al., 2013)
8	2-*O*-pentosyl-8-*C*-hexosyl-apigenin	37.557	195, 268, 334	563	$C_{26}H_{32}O_{15}$	503, 353, 325	ESI-MS PS-MS	*Celtis iguanaea* *Celtis iguanaea*	(Zanchet et al., 2018) (Almeida et al., 2024)

Retention time (Rt); High-Performance Liquid Chromatography with Diode-Array Detection and Tandem Mass Spectrometry (HPLC-DAD-MS/MS); High-Performance Liquid Chromatography coupled to Diode Array Detector and Electrospray Ionization Mass Spectrometry (HPLC-DAD-ESI-MS); Electrospray Ionization Mass Spectrometry (ESI-MS); Paper Spray Mass Spectrometry (PS-MS)

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Figures

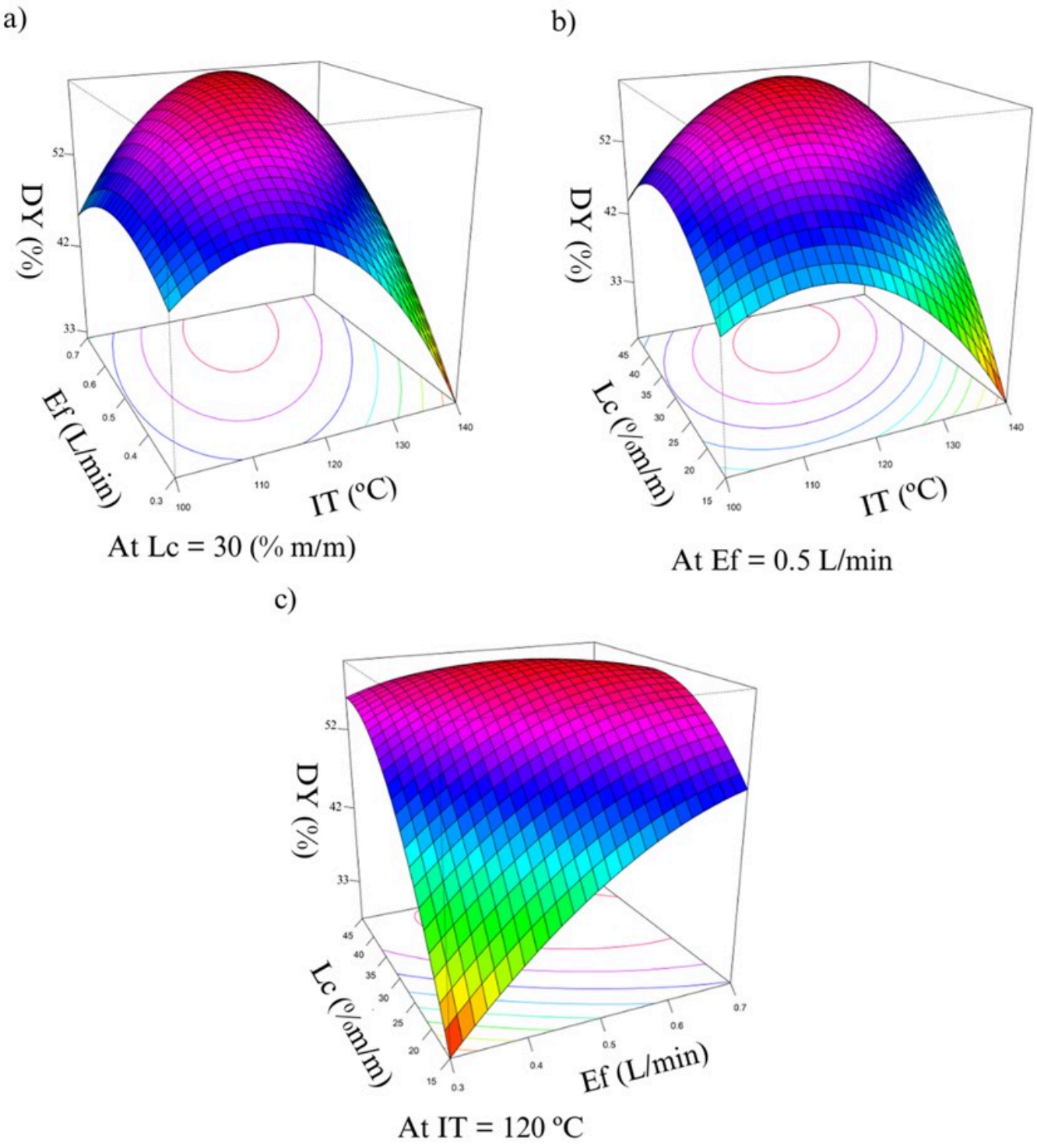

Figure 1

Response surface plots of the drying yield (DY) as a function of **(a)**drying air temperature (IT) and extract feed rate (Ef); **(b)** drying air temperature (IT) and leucine content (Lc); and **(c)** extract feed rate (Ef) and leucine content (Lc).

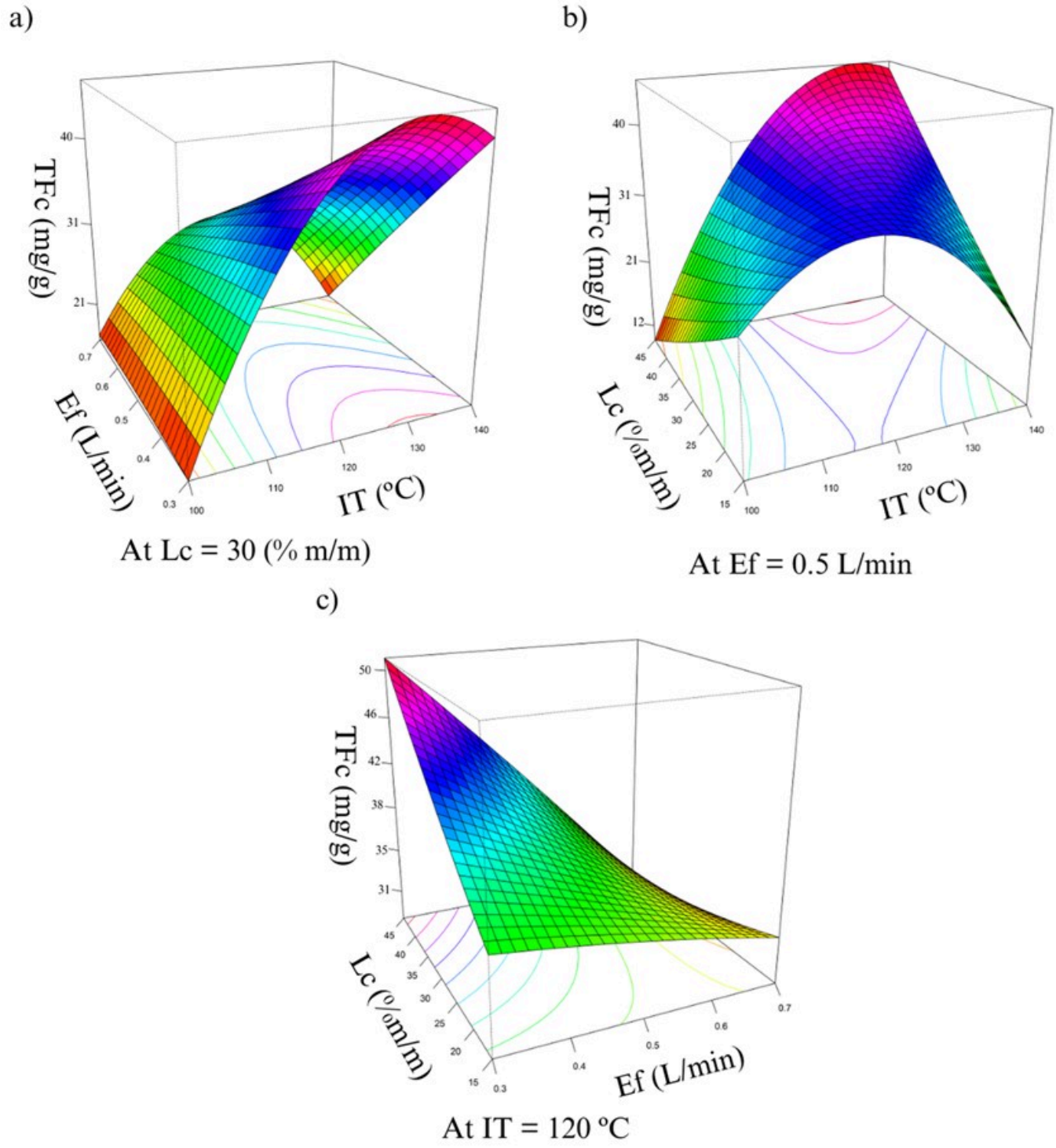

Figure 2

Response surface plots of the total flavonoid content (TFc) as a function of **(a)** drying air temperature (IT) and extract feed rate (Ef); **(b)** drying air temperature (IT) and leucine content (Lc); and **(c)** extract feed rate (Ef) and leucine content (Lc).

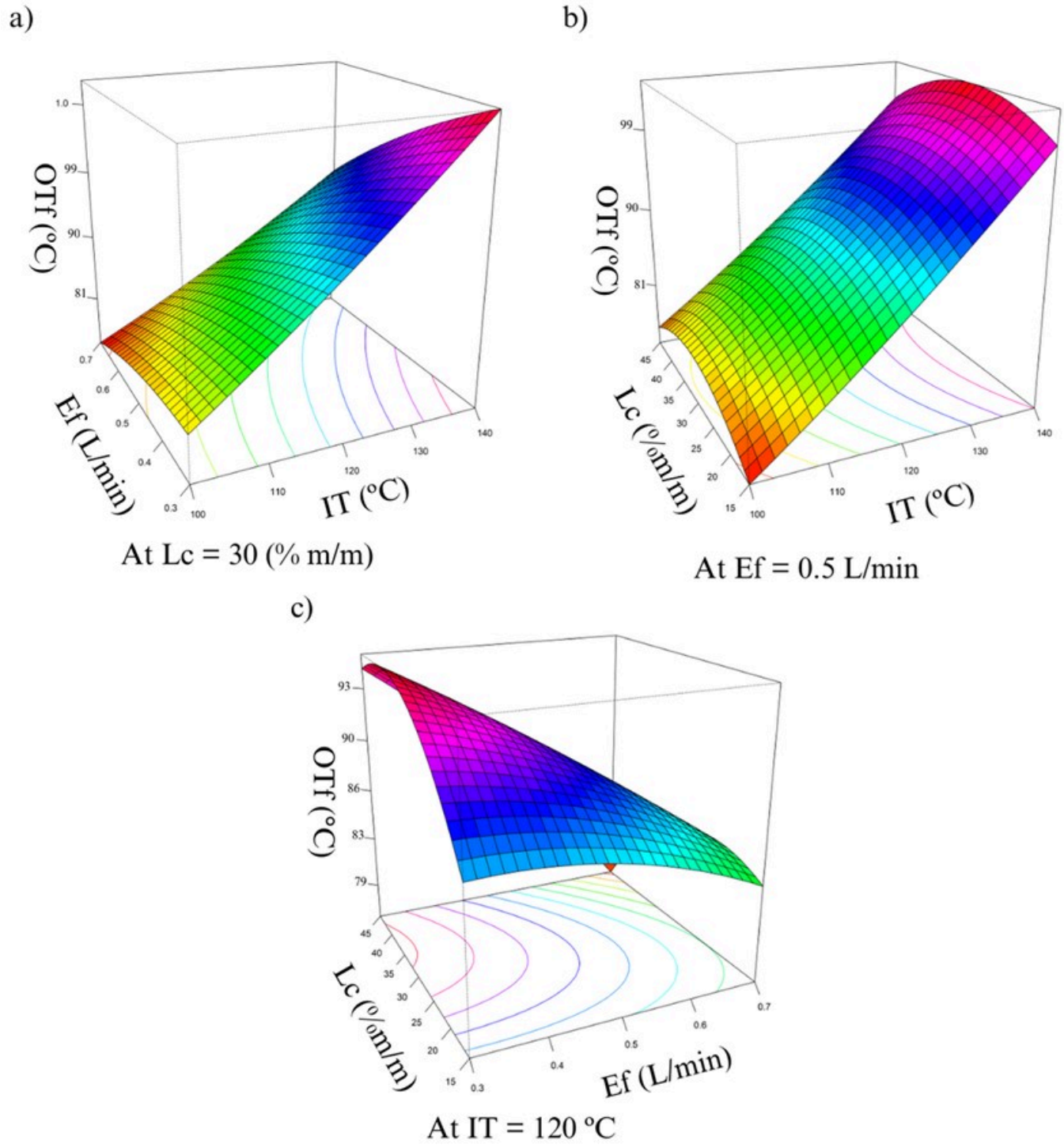

Figure 3

Response surface plots of the final drying air outlet temperature (Tf) as a function of **(a)** drying air temperature (IT) and extract feed rate (Ef); **(b)** drying air temperature (IT) and leucine content (Lc); and **(c)** extract feed rate (Ef) and leucine content (Lc).

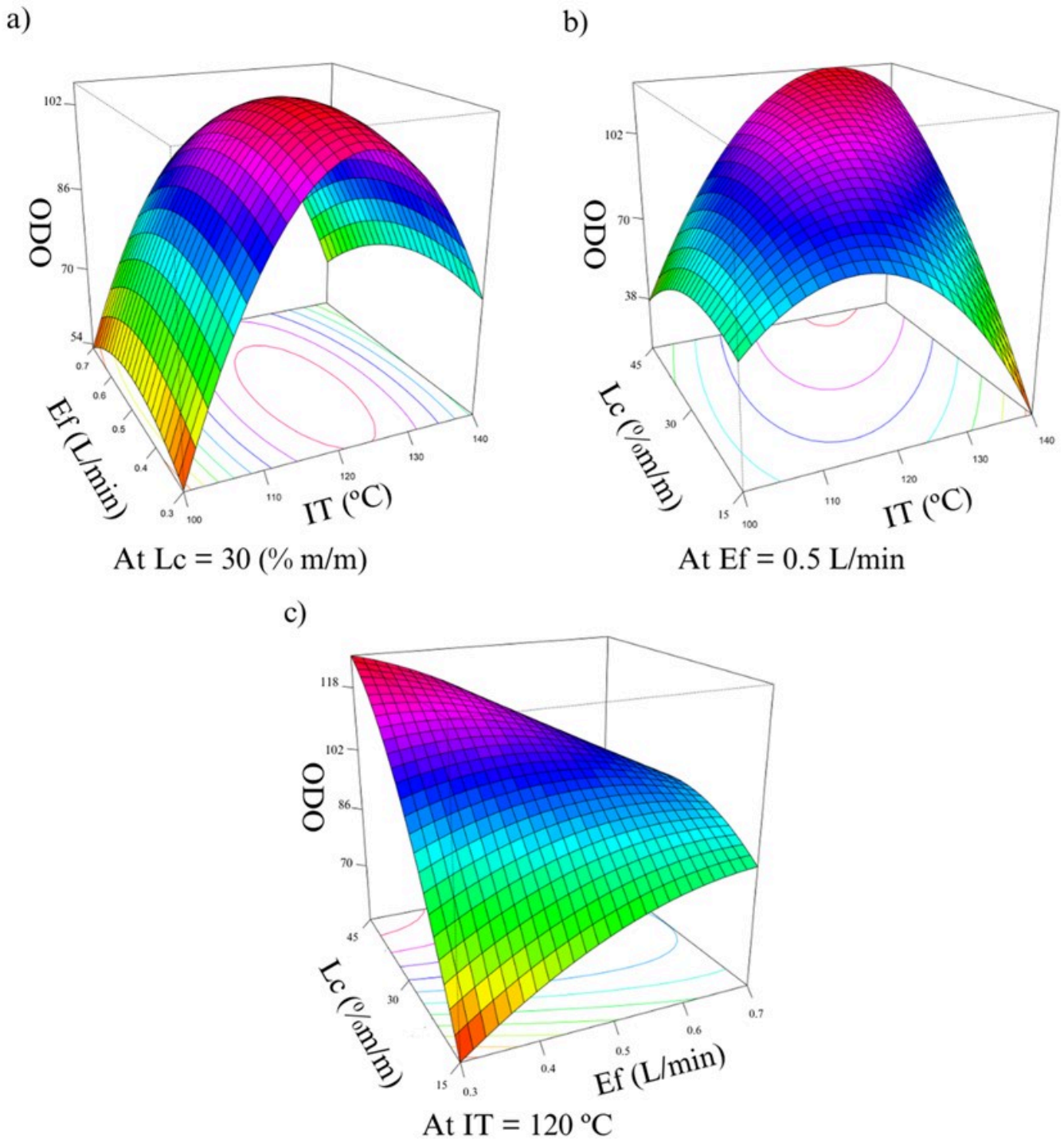

Figure 4

Response surface plots of the overall drying process optimization (ODO) as a function of **(a)** drying air temperature (IT) and extract feed rate (Ef); **(b)** drying air temperature (IT) and leucine content (Lc); and **(c)**extract feed rate (Ef) and leucine content (Lc).

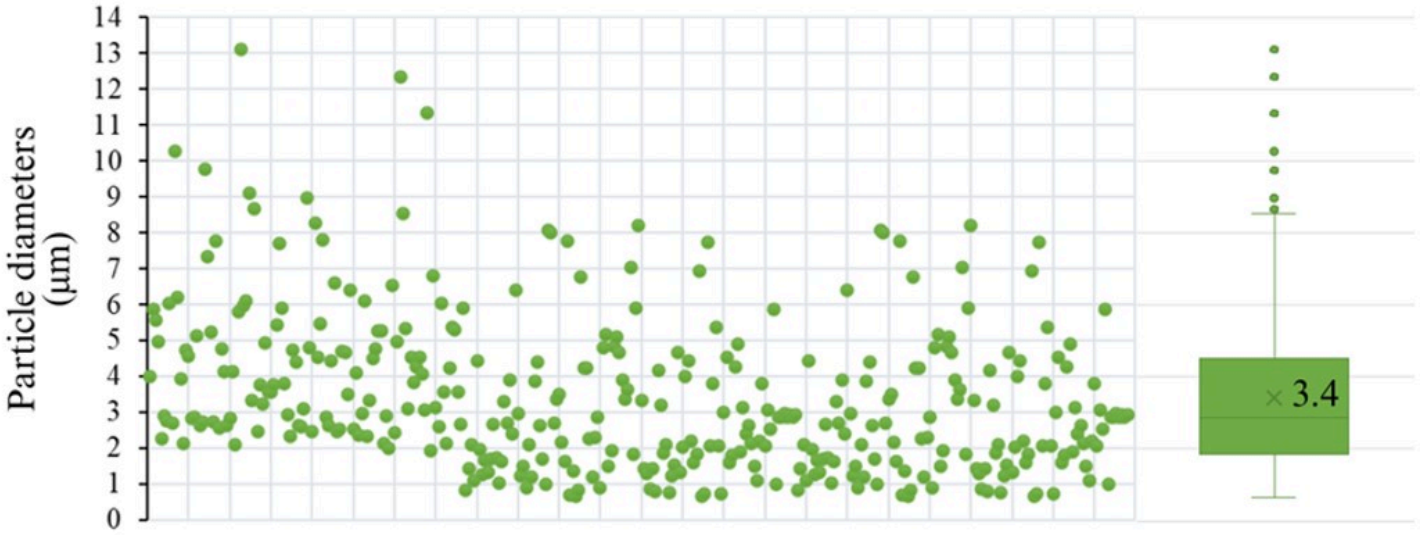

Figure 5

Records and histogram of particle diameters of the optimized *Celtis iguanaea* spray-dried extract (SDCi11)

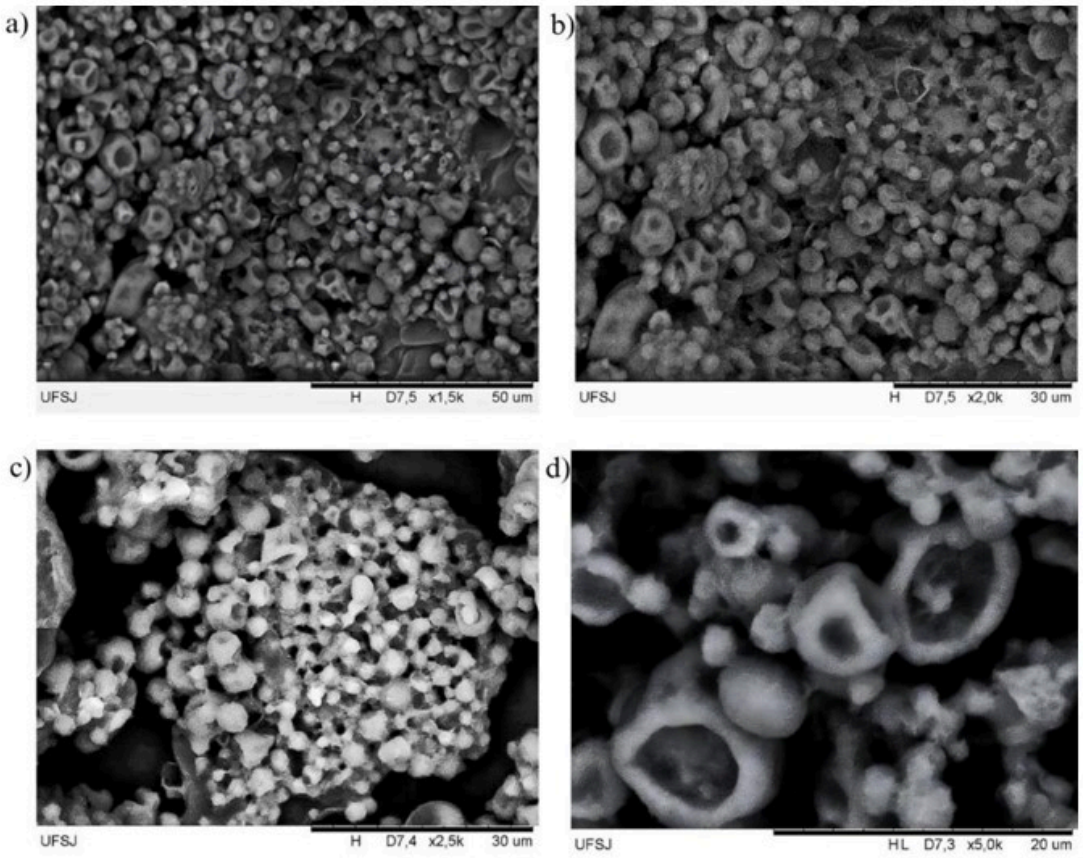

Figure 6

Scanning Electron Microscopy (SEM) images of the optimized *Celtis iguanaea* spray-dried extract (SDCi11) at magnifications of **(a)**1,500x; **(b)** 2,000x; **(c)** 2,500x; and **(d)** 5,000x.

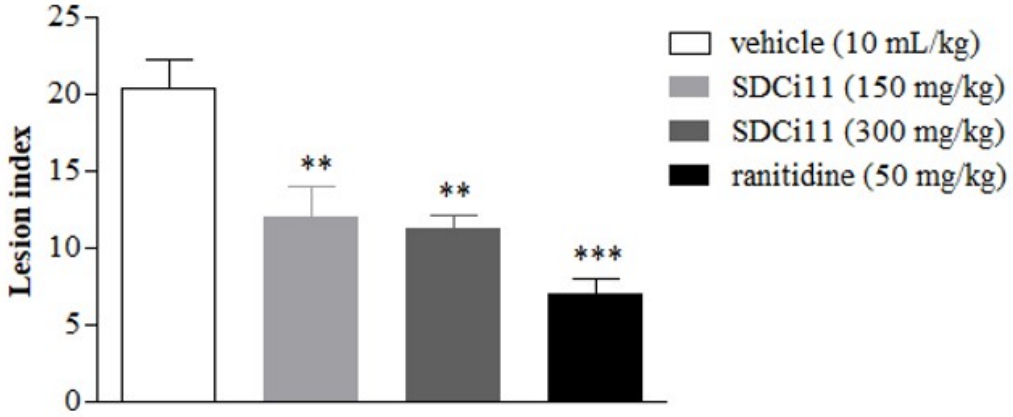

Figure 7

Effect of oral administration (p.o.) of the optimized *Celtis iguanaea* spray-dried extract (SDCi11) and ranitidine on the gastric lesion index in mice in the indomethacin-induced gastric ulcer model.

Data were expressed as mean ± SEM (n = 7) and analyzed by one-way ANOVA followed by the Newman-Keuls post-test. Significant at **p-value < 0.01 and *** p-value < 0.001 compared to the vehicle-treated group.

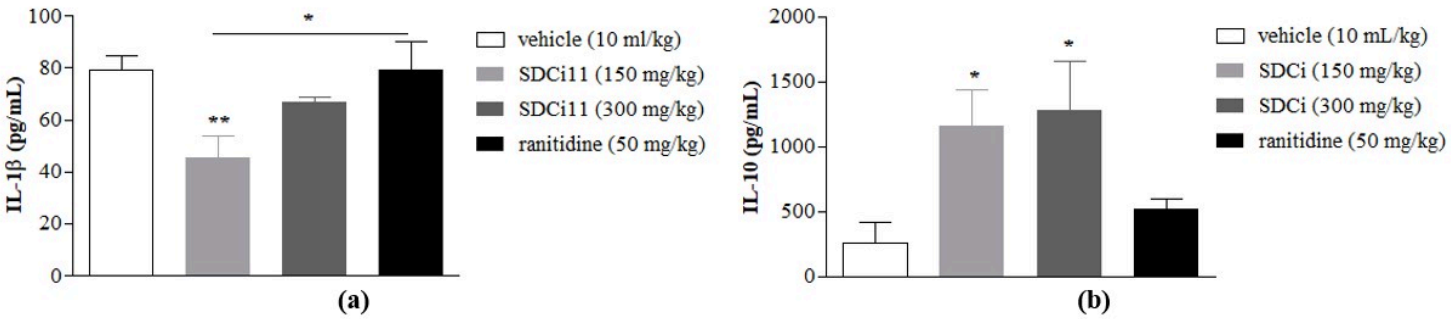

Figure 8

Effect of oral administration (p.o.) of the optimized *Celtis iguanaea* spray-dried extract (SDCi11) and ranitidine on indomethacin-induced changes in **(a)** IL-1β and **(b)** IL-10 levels in mouse stomachs.

Assays were duplicated and the results are expressed as mean ± SEM (n = 7) and analyzed by one-way ANOVA followed by the Newman-Keuls post-test. Significant at **p-value < 0.01 and * p-value < 0.05 compared to the vehicle-treated group or comparing SDCi11 and ranitidine in (a).

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