Assessment of composition and biological activity of *Arctium lappa* leaves extracts obtained with pressurized liquid and supercritical CO₂ extraction

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Abstract

Pressurized liquid extraction (PLE) of *Arctium* lappa leaves using aqueous ethanol is examined and presented for the first time in this work. In addition, global extraction yields, chemical composition, phenolic compounds and antioxidant activity of the PLE extracts were compared with those obtained by supercritical fluid extraction (SFE). PLE
extractions were evaluated at different conditions of pressure (15, 20 and 25) MPa and temperature (313.15, 333.15 and 353.15) K. The maximum global yield (37.40 wt %) was obtained by PLE at 15 MPa and 353.15 K. Furthermore, the PLE extracts contained higher concentrations of chlorogenic acid (1.84 %) and rutin (1.46 %) and exhibited considerably high DPPH free radical scavenging activity (160.54 µmol Trolox g⁻¹). PLE optimum extract exhibited considerable concentrations of phytol, lupeol and amyrin, making this a promising alternative for obtaining biologically active extracts from natural sources such as Arctium lappa leaves.

**Keywords:** Arctium lappa leaves; supercritical CO₂ extraction; pressurized liquid extraction; antioxidant activity; phenolic compounds.
1. **Introduction**

Due to the constant demand of the food and pharmaceutical industries for novel natural sources rich in nutritional and nutraceutical applications, researchers have been looking for the best ways to extract bioactive compounds from plant raw materials. One such example is the medicinal plant *Arctium lappa*, commonly known as burdock, considered in this study. *A. lappa* is a plant native to Europe and Asia, and it was rapidly spread to Brazil, where it grows spontaneously in fields, forests and rural areas. Its acclimatization is so natural that this plant is considered an invasive species [1,2].

The different parts of the burdock plant possess antioxidant [3–7], antibacterial [3,7–9] and anti-inflammatory biological activities [7,10,11]. Hence, its study is timely and feasible, moreover taking into consideration the ease and low cost of the plant's cultivation. Still, at present burdock is underused and there are just a few studies published devoted to recovery, characterization and application of its valuable extracts.

Bioactive compounds from *Arctium lappa* can be obtained by using different extraction methods, e.g. low pressure methods, such as Soxhlet, ultrasound and microwave extraction [6,12,13]. However, the focus at present is on extraction processes that use smaller amounts of solvents, present high extraction yields and have low environmental impact. In view of this, previous studies have examined the potential of a green solvent - supercritical carbon dioxide (scCO$_2$) - to obtain biologically active extracts from *A. lappa* [3,4], and the addition of ethanol as a co-solvent to change the non-polar nature of the solvent mixture and enhance the extraction of polar compounds [3,4,14–16].

Another extraction technique that has emerged as an alternative to the conventional ones is the pressurized liquid extraction (PLE). The PLE method uses
liquid solvents under high pressures and temperatures, normally above the boiling temperature. This favors extraction performance because the solvent remains in the liquid state even at temperatures above its boiling point [17].

High temperatures improve the mass transfer rates, favoring the extraction of solutes from the raw material to the solvent. Furthermore, raising the temperature increases the solubility and diffusivity of the compounds, while the surface tension and viscosity of the solvent are reduced. High pressure, in general, also favors the extraction because the solvent penetration throughout the structure of the solid matrix is facilitated and the solvent density is increased. Thus, the contact of the solvent with the compounds of interest is enhanced [17–19].

In this context, the main aim of this work was two-fold: i) to compare the capabilities to obtain extracts from *Arctium lappa* leaves, in terms of global extraction yield, of two high pressure extraction techniques, PLE and SFE and ii) to compare the quality of the extracts obtained in terms of antioxidant activity, chemical composition and total phenolic content. The results will reveal which of the two techniques performs better in terms of obtaining extracts rich in biologically active compounds.

2. Materials and Methods

2.1. Sample preparation and raw material characterization

The raw material (*Arctium lappa*) used in this work was the same used previously [3], where leaves and stems were collected in Ivaiporã (State of Paraná, Brazil). The aerial parts of the plants were harvested, sanitized and dried in an air circulation oven at
323.15 ± 2.0 K for 24 h. Then, the material was ground and granulometrically separated by Tyler series sieves. The average particle diameter and sample characterization were presented in a previous study [3].

2.2. Extraction methods

2.2.1. Sequential supercritical fluid extraction (SE)

The experiments were performed in a bench-scale supercritical extractor unit. The equipment and procedure applied in this study were described in details elsewhere [3,4,20–22]. As shown previously [3], extraction of A. lappa leaves with scCO$_2$, using ethanol as a co-solvent, worked well to obtain biologically active extracts. Thus, in this investigation, the scCO$_2$ with ethanol (scCO$_2$ + EtOH) was applied again but in a sequential steps extraction. The reason behind that was that in this way an assessment can be made which of the two applications performs better with respect to yield and extracts compositions. Six steps of sequential extractions were carried out with the view to guarantee complete recovery of the extractable material.

The extraction was performed under the optimum operating conditions identified previously, namely 15 MPa and 353.15 K [3], and at those conditions the same sample (the residue of a previous step) was subjected to another extraction with fresh solvents (scCO$_2$ and ethanol). For all steps the experimental procedure used was similar to that described in detail by Souza et al.[3]. Briefly, about 20 ± 0.2 g of the raw material was mixed beforehand with ethanol at a mass ratio of 2:1 (ethanol mass: mass of solids) and loaded into the extractor; next, the extractor was loaded with CO$_2$. The pressure and temperature were adjusted to the set point and the static extraction period was fixed at
60 min, after which the dynamic extraction (extraction period fixed at 25 min) was started using a compressed CO$_2$ with a flow rate around 2 mL min$^{-1}$. At the end of each step there was no ethanol residue in the extraction vessel. Besides that, to further examine the influence of co-solvents on SE efficiency, distilled water was added to ethanol as an auxiliary solvent (hereunder referred as scCO$_2$+Aq. EtOH procedure). For this, a hydroalcoholic solution containing water in ethanol at mass ratio of 2:8 (mass fraction of water 24 wt %), was prepared. This extraction was performed in only one step in order to be compared with the first step of the SE without adding water.

2.2.2. Pressurized liquid extraction (PLE)

The experiments were performed in a bench-scale pressurized liquid unit. The experimental system (Figure 1) consists of a buffer tank, a high-pressure positive displacement pump (Eldex, 2SM Optos Series – São Paulo, SP, Brazil), a needle valve (Parker, MVE Series – São Paulo, SP, Brazil) to stop the flow, a pre-heating vessel, a stainless steel extraction cell (inner volume $V = 1.8 \times 10^{-5}$ m$^3$, length $L = 0.16$ m, diameter $\Phi = 1.2 \times 10^{-2}$ m) with heating jacket for temperature control, followed by a chiller vessel coupled to a temperature controlled bath (Solab, SL 152 – Piracicaba, SP, Brazil). For trol in the extractor a needle valve (Parker, MVE Series– São Paulo, SP, Brazil) and a back-pressure regulator (Swagelok, KPB Series – São Paulo, SP, Brazil) were used. All pipes and connections were of stainless steel (1/8”). The PLE was performed with aqueous ethanol (water content fixed at 12 wt %) as solvents. About 7.5 g of raw material was used, thus creating a fixed bed inside the extractor (18 mL). The experimental procedure started by adjusting the extraction vessel temperature. After the system reached the temperature required, the needle valve at the exit of the extraction
cell was opened so that the solvent, when pumped at a given flow rate (normally 2.0 mL min⁻¹), could reach the extraction cell. When the solvent was visualized in the pipe output, the needle valve and the back-pressure regulator were closed thus allowing the system to reach the desired pressure, which was controlled (using the back-pressure regulator) at the set point. At the end of the static extraction time (10 min) equilibrium was reached, thus the needle valve was opened, the pressure was regulated using the back-pressure regulator, and the dynamic extraction started. Preliminary tests with different water content in ethanol were performed (as described in section 3.2, Table 2), which showed that total phenolic compounds and the highest extraction yields were achieved with aqueous ethanol at the mass ratio of 1:9 (mass fraction of water 12 wt %). Hence, this aqueous ethanol solution was used in all subsequent experiments.

Furthermore, to assess the influence of temperature and pressure on the extraction yield, experiments were carried out at temperatures of (313.15, 333.15 and 353.15) K and pressures (15.0, 20.0 and 25.0) MPa, following a randomized 2² experimental design with a triplicate at the central point. These pressure and temperature levels were selected based on the previous study [3], thus allowing the comparison of biological activities for the extracts obtained from different techniques at same levels of temperature and pressure. The maximum temperature was fixed at 353.15 K in order to prevent decomposition of the heat sensitive compounds present in the extracts. The mass fraction of water in the hydroalcoholic solution was fixed at 12 wt %. The solvent flow rate was (1.2 ± 0.15) mL min⁻¹. The extraction time was 240 min, defined by preliminary tests where a plateau of yield was reached. The overall extraction curves (extraction kinetics) were obtained by collecting and weighing the extract samples at different times. The samples collected were dried in an air circulation
oven to remove solvent. The extracts were stored in the dark and under low temperature (268.15 ± 2.0 K) until analyzed.

2.3. Gas chromatography with mass spectrometry analysis (GC/MS)

In order to be analyzed, the extracts were dissolved (1 mg mL⁻¹) in dichloromethane (spectroscopic grade, 99.9% purity, Sigma-Aldrich). The equipment used was GC-MS (Shimadzu TQ8040 – Barueri, SP, Brazil), consisting of a HP-5MS non-polar capillary column (30 m × 0.25 mm di, thickness of 0.25 μm, 5% phenyl / 95% dimethylpolysiloxane). The experimental procedure applied was similar to that described by Souza et al. [3]. Compounds identification was based on the NIST-14 library database, and on comparison with data available in the literature.

2.4. Antioxidant Activity Evaluation

2.4.1. Total phenolic content (TPC)

The total phenolic content (TPC) was determined according to the Folin–Ciocalteu method [23], using gallic acid as a standard, as presented previously [3]. The procedure consisted of mixing 0.1 mL of extract (6 mg mL⁻¹), 7.9 mL of distilled water, 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of 20 % sodium carbonate, in volumetric flasks. The reaction mixture was kept in the dark for 2 h and then the absorbance was measured.
at 765 nm. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract (mg GAE/g of extract).

2.4.2. Antioxidant activity by DPPH assay

DPPH assay was performed, following Souza et al. [3], using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, where the procedure was adapted from Mensor et al. [24]. The results were expressed as the half-maximal inhibitory concentration (IC$_{50}$), where IC$_{50}$ is the concentration (mg mL$^{-1}$) of extract required to inhibit the production of radicals by 50 %, and also given in μmol trolox equivalent g$^{-1}$.

2.4.3. Antioxidant activity by FRAP assay

Antioxidant activity was determined according to the procedure described by Barbi et al. [25], according to the method proposed by Benzie and Strain [26]. The results were expressed in μmol trolox equivalent g$^{-1}$.

2.4.4. Antioxidant activity by ABTS assay

The method of Re et al. [27] was used to determine the ABTS$^{++}$ scavenging activity, according to the procedure described by Barbi et al. [25]. The results were expressed in μmol trolox equivalent g$^{-1}$.

2.5. Phenolic Compounds Identification
A reversed phase HPLC method was employed to determine the concentrations of gallic acid (3,4,5-trihydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), chlorogenic acid (5-O-caffeoylquinic acid) and rutin hydrate (quercetin-3-rutinoside hydrate) in the *A. lappa* leaves extracts. Samples were solubilized with acetonitrile (Panreac – HPLC grade) and eluent A (mobile phase described in the sequence), and analyzed applying Agilent 1200 Series HPLC system (Wilmington, DE, USA), equipped with a diode arrangement detector (DAD) commanded by Software EZChrom Elite (Agilent), an automatic liquid sampler (ALS), and quaternary pump. The compounds were separated by Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm) (Agilent). The injection volume was 15 µL. The mobile phase consisted of water (ultrapure), formic acid (vetec 80 % v/v) and acetonitrile (95:2:3, v/v/v; eluent A) and water/formic acid/acetonitrile (48:2:50, v/v/v; eluent B). Analyses were conducted using a gradient with eluent A and B, programmed as follows: the run started from 88 % A and changed to 85 % A (6 min), to 60 % A (16 min), to 53 % A (20 min), to 15 % A (24 min), maintained for up to 31 min, and returned to 88 % A (32 min) up to the end of the run (35 min). The flow rate was constant at 0.9 mL min⁻¹. Chromatograms were recorded at 280 nm for gallic acid and 3,4-dihydroxybenzoic acid determination. The chlorogenic acid and rutin were determined at 320 and 370 nm, respectively. The concentration of compounds was determined by external calibration using standards (purity > 99 %) purchased from Sigma-Aldrich. The identification of compounds was carried out on the basis of retention time and UV absorption spectrum of the standards.

Results obtained in this study were statistically analyzed using Statistica 7.0® (Analytical Software, Tallahassee, FL, USA), as described by Souza et al. [3].
3. Results and Discussion

3.1. Supercritical fluid extraction (scCO$_2$)

The characteristics of the raw material used in this study were given in a previous work [3]. Dry *A. lappa* leaves had residual moisture of about 5.97 ± 0.02 wt %, and mean particle diameter, defined by the Tyler series, of $(1.3 ± 0.4) \times 10^{-3}$ m [3].

For the SEs the same sample was used for all six steps, while in the scCO$_2$+Aq. EtOH procedure fresh raw material was used, for only one step extraction. In all experiments the extractions were performed at 15 MPa, 353.15 K and with ethanol to solids mass ratio of 2:1 (ethanol mass: mass of solids). The antioxidant activity was evaluated to verify biological activities variations in the extracts obtained. The extraction yields applying scCO$_2$+EtOH in consecutive steps, and the yield obtained by the scCO$_2$+Aq. EtOH procedure are presented in Table 1, where the antioxidant activity and phenolic content results are shown as well.

Figure 2 shows the overall extraction curves, where the total yield at the end of the sixth step (150 min) reached 12.8 wt % (Run 6), while in a single step, after 25 min of extraction, the yield was about 6 wt % (Run 1). In the sequential extraction (Runs 1-4), the phenolic content in the extracts was significantly changed only for step 4 and the antioxidant activity verified by DPPH method showed a decrease in the IC$_{50}$ value from the first to the second step, indicating a small improvement in this biological property. Although the SE achieved a higher yield and extracts with good biological properties, the solvent volume used was very large, approximately five times greater than in the single step extraction. From this point of view, the application of the sequential
extraction method might not present great advantages over the single step extraction for this particular raw material.

However, it was found out that for the single step scCO$_2$+Aq. EtOH procedure, it was possible to achieve about 9 wt % yield in just 25 min of extraction (Run 7), which was an increase of about 3 wt % as compared to the yield obtained for the same time of extraction using ethanol as a co-solvent. To achieve good efficiency in a short time is very important for energy savings [28]. The same phenomena was observed by Solana et al. [29] and Paes et al. [30]. As expected, the higher yield results for scCO$_2$ were obtained when water was introduced in the system with the co-solvent. In addition to good extraction yields, the extract obtained contained a high concentration of phenolic compounds (72.32 mg GAE g$_{extract}^{-1}$) when compared to the other ones, probably due to the hydrophilic nature of constituents, such as rutin hydrate [31].

Regarding the extraction using scCO$_2$+EtOH (or Aq. EtOH), it should be noted that, as discussed in [3], in these extractions basically a switch of solvents inside the extractor takes place: starting with CO$_2$-expanded ethanol and ending the extraction with pure CO$_2$. The kinetic and thermodynamic aspects of this kind of process are very interesting and are a topic of a future research.

Figure 2

3.2. Pressurized liquid extraction (PLE)

Among extraction methods, PLE has stood out due to using smaller amounts of solvent [32,33]. In our research, the PLE was used as an alternative technique for obtaining extracts of A. lappa leaves with biological activities. The preliminary tests revealed that
the best extraction conditions were at $T = 353.15$ K and $p = 15$ MPa, respectively. Table 2 shows the global yield, phenolic content and antioxidant activity of the extracts obtained by PLE performed with different hydroalcoholic concentrations.

Initially, to compare the efficiency of PLE with Soxhlet extraction carried out with water as a solvent, the extractions were carried out with different mass fractions of distilled water in the hydroalcoholic solution (12, 24, 55 wt %). Though it was possible to verify that the addition of water promoted an increase in the extraction yield (Run 1 and 2), still, there was not a significant increase observed for the different hydroalcoholic concentrations (Runs 2-4). In a previous study, Souza et al. [3] showed that the extraction yield of A. lappa leaves is improved in the presence of a polar solvent, indicating that the constituents of this raw material are mostly polar compounds. Therefore, the addition of water to the solvent in PLE resulted in obtaining a maximum extraction yield [34]. TPC values were also improved in the presence of water because, generally, it enhances phenolics extraction [33].

From the results obtained, it can be seen that water addition improved both extraction yield and TPC values, while the antioxidant activities decreased. Therefore, the mass fraction of water in the hydroalcoholic solution was fixed at 12 wt %, and a randomized $2^2$ experimental design was carried out to determine the influence of temperature and pressure.

The different experimental conditions applied to obtain extracts of A. lappa by PLE using the above hydroalcoholic solution are presented in Table 3, where the global yield, phenolic content and antioxidant activity are shown as well.

Table 3 demonstrates that all extraction yield values are higher than those obtained by SE (Table 1). It should be noted that in the extractions performed at a fixed temperature (Runs 1-2 and 3-4, respectively) the extraction yield decreased with
increasing the pressure, as shown in Figure 3 (a-b). However, at a fixed pressure an increase in the temperature (Runs 1-3 and 2-4, respectively) enhanced the extraction yield, as shown in Figure 3 (c-d). From Figure 3, it can be deduced that within the range of the conditions investigated the effect of the temperature is more pronounced than that of the pressure. At a constant pressure, the temperature increase contributes to increasing the solubility of solutes in ethanol. However, even though the density of ethanol is increasing, the pressure increase might be affecting other physical-chemical parameters, such as viscosity and surface tension, thus leading to lower kinetics and mass transfer coefficients. Furthermore, the highest pressure applied in the experiments might be also promoting a high bed packing thus decreasing the rate of extraction related to the constant extraction rate (CER) period. It is worth mentioning that the PLE extraction has not reached the step of extraction controlled by diffusion, related to the FER period (falling extraction rate). Thus, further studies aiming to reach the FER period should be performed in order to better understand the kinetic behavior of burdock leaves extraction by PLE method.

**Figure 3**

Statistically, the PLE process was influenced significantly \( (p < 0.05) \) by both the temperature and pressure variables, as shown in the Pareto chart (Figure 4a), while Figure 4b shows the corresponding response surface in terms of extraction yield. The highest yield (37.4 wt %) was obtained at 15 MPa and 353.15 K (Run 3), and the extract exhibited the highest phenolic content \( (55.38 \, \text{mg}_{\text{GAE}} \, \text{g}_{\text{extract}}^{-1}) \) and lowest IC\textsubscript{50} \( (0.20 \, \text{mg}_{\text{extract}} \, \text{ml}^{-1}) \), indicating the best antioxidant capacity.
3.3. Phenolic compounds identification and antioxidant activity.

As the results of TPC indicated, the *A. lappa* leaves extracts contain high concentration of phenolic compounds, and of these, analogously to Solana et al. [29], rutin and some phenolic acids were identified in this study. Table 4 shows that the compounds present in higher amounts in all the samples were chlorogenic acid and rutin, while gallic and dihydroxybenzoic acids are in lower concentrations. Sample comparison shows that the higher phenolic concentrations are exhibited by the extracts obtained by the PLE and seCO$_2$+Aq. EtOH procedure (Runs 1-5 and Run 9). As expected, the presence of water positively influenced the extraction of phenolic compounds by both techniques (according to the results displayed in Table 1 and Table 2, the water presence improved the TPC values). Still, in the extracts obtained by PLE, the phenolic concentration was higher, with the best values for chlorogenic acid and rutin identified in the samples obtained at the highest temperature (353.15 K) and at pressures of 15 MPa and 25 MPa (Runs 3 and 4, respectively), which is in agreement with the results obtained and presented for the TPC values (Table 3). This fact reinforces the observation made previously that in the PLE process the temperature is the main factor that positively influences the extraction. The same behavior was verified in other studies as well, see for example Pereira et al. [33] for grape marc extracts, Garcia-Mendoza et al.[32] for juçara residues extracts, and Manuel et al. [35] for citrus products extracts. Possibly, this is due to an increase in the solubility and diffusivity at high temperatures of the phenolics in the mixed solvent (ethanol + water), in addition to the decrease in the viscosity and other factors, which might influence the extraction efficiency [17,36].
In order to ascertain whether the extraction process can influence the biological properties of the extracts obtained, the antioxidant capacity was measured as well. The data obtained is presented in Table 4 and Figure 5, where it is clearly demonstrated that the antioxidant results are related with the phenolic compounds, as obtained by Ferrentino et al. [37]. Figure 5 displays the linear correlation between antioxidant capacity (expressed as IC_{50}) and total phenolic compounds (TPC) for the burdock leaves extracts obtained by PLE, which is in complete analogy with the results presented in the previous work using scCO_{2} + EtOH [3]. In general, all extracts exhibited antioxidant properties. For the PLE (Runs 1-5), the extract obtained at lower pressure and higher temperature (15 MPa and 353.15 K, Run 3) exhibited the best results, considering all three assays (DPPH, ABTS and FRAP). It also showed higher phenolic compounds concentrations (mainly chlorogenic acid and rutin), which is in agreement with the observation that these compounds show better results with the three assays [38,39]. However, a more thorough examination reveals that for both techniques (PLE and SFE) the best results varied according to the different antioxidant assay used. As discussed by Shibamoto and Moon [38], variations in the antioxidant properties obtained by applying DPPH, ABTS and FRAP are observed because some of the compounds present in the extracts exhibit similar UV wavelength maxima as the reactant compounds, thus causing an overall interference in the results. Therefore, it is recommended to use different methods to investigate the antioxidant activities of the samples. In general, taking into account the phenolic compounds concentrations as well as the antioxidant tests results, it is possible to conclude that the sample that exhibited the best responses was the extract obtained via PLE under 15 MPa and 353.15 K (Run 3).
3.4. Chemical Composition

In the *A. lappa* leaves extracts, different compounds were identified by chemical composition analysis - e.g. terpenoids, phenol and esters. Their relative compositions (%) are presented in Table 5.

For the extracts obtained by PLE and SFE with scCO$_2$, the compound that has the highest concentration is lupeol (4-36%). Analogous results were obtained by Souza et al. [3]. Lupeol is a triterpene naturally present in some vegetable matrices and has gained attention because of its high biological effects like anti-inflammatory and anticancer [40–44]. 2,4-di-tert-butyl phenol found in some samples is also of significant importance, because of its beneficial antioxidant and antimicrobial properties [45–47]. The high concentrations of phytol and amyrin determined were in complete analogy to the results obtained previously [3]. Phytol is a diterpene that has antioxidant and anti-inflammatory properties [48]. Amyrin is a triterpene, which possesses important biological and pharmacological properties [49,50] such as gastroprotective [51], anxiolytic, antidepressant [52], and antinociceptive [53], as well as antioxidant and cytotoxicity activities [54,55], characterizing these extracts with biological activity potential.

In general, it can be confirmed that the extraction techniques and operating conditions applied influenced substantially the extracts composition. For example, the compounds of interest are present in higher concentration in the extracts obtained when scCO$_2$ was used as a solvent (samples 6-9). Yet, even though the PLE extracts exhibited lower concentrations of the compounds of interest in the overall results, in terms of extraction yield, antioxidant activities and total phenolics the results were quite
satisfactory. Hence, it can be concluded that the PLE is a viable approach to obtaining extracts with high biological activities from burdock leaves.

4. Conclusions

The present work investigates the potential of different methods for extracting biologically active compounds from *A. lappa* leaves. PLE and scCO$_2$ are environmentally friendly alternatives to conventional techniques, which proved to be effective and viable for obtaining extracts of *A. lappa* leaves with good biological properties, thus encouraging their use in other applications. Although all extracts obtained by the different methods examined exhibited biological properties, the PLE showed the highest extraction yield (37.40 wt %), while the sequential scCO$_2$+EtOH and the scCO$_2$+Aq. EtOH procedure achieved 12.78 wt % and 9.12 wt %, respectively. Furthermore, the PLE presented an advantage over the other techniques because in addition to the higher yield, it showed good results for the recovery of bioactive compounds from *Arctium lappa* leaves, supported by the high concentrations of phenolic compounds, such as chlorogenic acid and rutin hydrate, and high antioxidant activities. The PLE with a water solvent (a hydroalcoholic solution) proved to be a promising option in terms of increasing the global yield, phenolic compound concentration and antioxidant activity.

Temperature demonstrated a positive influence on the process, and the best overall results were obtained at the highest temperature applied, 353.15 K and 15 MPa, because increase in the temperature improves the diffusion and solubility of the phenolic compounds in the different solvents, and hence promotes their recovery.
Also, positive correlations between TPC and antioxidant activity were observed for the PLE extracts. In summary, the advantages of PLE substantiate its application for the extraction of compounds of interest from plant matrices such as *A. lappa*.

In this work, for the first time, the capabilities of PLE technique to obtain bioactive compounds with high antioxidant activity from burdock leaves were presented. In addition, the results obtained can serve as an impetus to cultivate and further explore an easily available and low-cost raw material.

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Figure Captions

Figure 1. PLE experimental system: (1) buffer tank; (2) high-pressure positive displacement pump; (3) on-off valve; (4) pre-heating vessel; (5) extraction cell with temperature control; (6) chiller vessel; (7) temperature controlled bath; (8) manometer; (9) needle valve; (10) back-pressure regulator; (11) collecting container of the final extract; (12) pressure and temperature sensors.

Figure 2. Extraction curves of six step SE using ethanol as co-solvent at 15 MPa and 353.15 K: (□) step 1; (■) step 2; (∆) step 3; (●) step 4; (◊) step 5; (★) step 6.

Figure 3. Effect of temperature and pressure on the PLE yield at either fixed temperature in (a) 313.15 K and (b) 353.15 K, or fixed pressure in (c) 15MPa and (d) 25 MPa.

Figure 4. Statistical analysis of the PLE results: (a) Standardized Pareto charts for the response variables temperature and pressure studied in the experimental design and (b) corresponding response surface.

Figure 5. Comparison between antioxidant capacity (IC₅₀) and total phenolic compounds (TPC) for the burdock leaves extracts obtained by PLE technique.
Figure 1

Figure 2
Figure 3
Figure 4
Figure 5
Table 1. Extraction yields, and antioxidant capacity determined by DPPH method for *A. lappa* leaves extracts obtained at 15 MPa and 353.15 K using scCO$_2$+EtOH in SE and with addition of water (scCO$_2$+ Aq. EtOH).

| Run | Extraction | Extraction Total Time (min) | Individual yield (wt%) | Cumulative yield (wt%) | TPC (mg GAE g$_{extract}^{-1}$)$^#$ | IC$_{50}$ (mg$_{extract}$ mL$^{-1}$)$^#$ |
|-----|------------|-----------------------------|------------------------|------------------------|-----------------------------------|-----------------------------------|
| 1   | scCO$_2$SE step 1 | 25                          | 6.10 ± 0.22            | 6.10 ± 0.22            | 44.57$^{a,b}$ ± 0.8               | 0.40$^a$ ± 0.03                   |
| 2   | scCO$_2$SE step 2 | 50                          | 2.79 ± 0.17            | 8.89 ± 0.06            | 42.93$^{a,b}$ ± 3.91             | 0.24$^b$ ± 0.01                   |
| 3   | scCO$_2$SE step 3 | 75                          | 1.75 ± 0.55            | 10.65 ± 0.50           | 47.62$^b$ ± 0.91                 | 0.21$^b$ ± 0.03                   |
| 4   | scCO$_2$SE step 4 | 100                         | 0.88 ± 0.07            | 11.53 ± 0.43           | 39.21$^b$ ± 2.77                 | i.e.                              |
| 5   | scCO$_2$SE step 5 | 125                         | 0.74 ± 0.07            | 12.27 ± 0.35           | i.e.                             | i.e.                              |
| 6   | scCO$_2$SE step 6 | 150                         | 0.50 ± 0.12            | 12.78 ± 0.47           | i.e.                             | i.e.                              |
| 7   | scCO$_2$+Aq. EtOH | 25                          | 9.12                   | 9.12                   | 72.32 ± 3.17                     | 0.70 ± 0.03                       |

i.e. – Insufficient extract
$^#$ Different letters indicate significant differences by the Tukey’s test (p < 0.05)

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Table 2. Extraction yields, phenolic content and antioxidant capacity determined by DPPH method for *A. lappa* leaves extracts obtained by PLE carried out at 15 MPa and 353.15 K, in the fixed extraction time at 240 min, using hydroalcoholic solution with different mass fraction of water.

| Run | Mass fraction of water (wt%) | Extraction Yield (wt%) | TPC (mg GAE g$_{extract}^{-1}$)$^#$ | IC$_{50}$ (mg$_{extract}$ mL$^{-1}$)$^#$ |
|-----|-------------------------------|------------------------|-----------------------------------|-----------------------------------|
| 1   | 0                             | 13.50$^c$              | 42.04$^c$ ± 1.15                  | 0.28$^c$ ± 0.005                  |
| 2   | 12                            | 37.40$^a$              | 55.38$^b$ ± 2.18                  | 0.20$^d$ ± 0.006                  |
| 3   | 24                            | 39.43$^a$ ± 0.98$^S$  | 37.45$^c$ ± 1.03                  | 0.28$^c$ ± 0.005                  |
| 4   | 55                            | 40.35$^a$              | 69.03$^a$ ± 2.98                  | 0.38$^b$ ± 0.020                  |
| 5*  | 100                           | 33.58$^b$              | 81.55$^d$ ± 6.60                  | 0.67$^a$ ± 0.002                  |

*Soxhlet extraction with water solvent.
$^S$ Standard deviation from a replicate experiment and assumed the same value for all conditions.
$^#$ Different letters indicate significant differences by the Tukey’s test (p < 0.05)

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Table 3. Extraction yields, phenolic content and antioxidant capacity determined by DPPH method for PLE of *A. lappa* leaves extracts at different temperatures and pressures using hydroalcoholic solution (mass fraction of water 12 wt %) at 240 min of extraction time.

| Run | $P$ (MPa) | $T$ (K) | Extraction Yield (wt%) | TPC (mg$_{GAE}$/g$_{extract}$) | IC$_{50}$ (mg$_{extract}$ mL$^{-1}$) |
|-----|-----------|---------|------------------------|--------------------------------|-----------------------------------|
| 1   | 15        | 313.15  | 22.41                  | 13.14$^d$ ± 1.38               | 0.63$^b$ ± 0.10                   |
| 2   | 25        | 313.15  | 17.86                  | 23.28$^c$ ± 1.50               | 0.46$^c$ ± 0.04                   |
| 3   | 15        | 353.15  | 37.40                  | 55.38$^a$ ± 2.18               | 0.20$^a$ ± 0.06                   |
| 4   | 25        | 353.15  | 28.10                  | 41.64$^b$ ± 2.06               | 0.25$^d$ ± 0.09                   |
| 5*  | 20        | 333.15  | 26.61 ± 1.42           | 14.46$^d$ ± 3.41               | 0.53$^b$ ± 0.03                   |

*Central point for the randomized $2^2$ experimental design.
$^#$ Different letters indicate significant differences by the Tukey’s test (p < 0.05)
Table 4. Phenolic Compound identified and Antioxidant Activity results for the A. lappa leaves extracts obtained by different extraction methods: PLE using hydroalcoholic solution (mass fraction of water 12 wt %) , SE in different steps using ethanol as a co-solvent and SFE using hydroalcoholic solution (mass fraction of water 24 wt%) as co-solvent.

| Run | Extraction | P (MPa) | T (K) | Gallic Acid | Dihydroxybenzoic Acid | Chlorogenic Acid | Rutin Hydrate | IC$_{50}$ (mg$_{extract}$ mL$^{-1}$) | DPPH (µmol$_{Trolox}$ g$^{-1}$) | FRAP (µmol$_{Trolox}$ g$^{-1}$) | ABTS (µmol$_{Trolox}$ g$^{-1}$) |
|-----|------------|---------|-------|-------------|-----------------------|-----------------|--------------|---------------------------------|-------------------------------|-------------------------------|-------------------------------|
| 1   | PLE        | 15      | 313.15| 0.1205      | 0.0596                | 1.1125          | 0.6423       | 0.63$^a$ ± 0.10                  | 102.55$^e$ ± 1.78             | 63.95$^{de}$ ± 1.16           | 165.20$^a$ ± 0.85             |
| 2   | PLE        | 25      | 313.15| 0.0974      | 0.0533                | 0.9827          | 0.6341       | 0.46$^c$ ± 0.04                  | 115.14$^d$ ± 2.06             | 48.61$^f$ ± 1.09              | 170.92$^{de}$ ± 0.28          |
| 3   | PLE        | 15      | 353.15| 0.0908      | 0.0716                | 1.8453          | 1.4656       | 0.19$^c$ ± 0.06                  | 160.54$^b$ ± 3.07             | 66.51$^d$ ± 2.08              | 202.30$^b$ ± 3.00             |
| 4   | PLE        | 25      | 353.15| 0.1003      | 0.0610                | 1.3068          | 1.2868       | 0.25$^d$ ± 0.09                  | 115.31$^d$ ± 2.39             | 45.83$^f$ ± 0.54              | 151.47$^{f}$ ± 1.76           |
| 5*  | PLE        | 20      | 333.15| 0.1142      | 0.0401                | 0.8726          | 0.8952       | 0.53$^b$ ± 0.03                  | 130.10$^c$ ± 1.53             | 60.49$^e$ ± 3.74              | 174.18$^d$ ± 2.04             |
| 6   | scCO$_2$-SE step 1 | 15 | 353.15 | 0.0384 | 0.0227 | 0.2899 | 0.3549 | 0.40$^c$ ± 0.03 | 104.59$^e$ ± 0.51 | 86.29$^c$ ± 3.01 | 191.34$^e$ ± 1.13 |
| 7   | scCO$_2$-SE step 2 | 15 | 353.15 | 0.0709 | 0.0319 | 0.4597 | 0.6307 | 0.24$^b$ ± 0.01 | 135.20$^{bc}$ ± 1.76 | 110.33$^b$ ± 0.83 | 170.42$^{de}$ ± 1.72 |
| 8   | scCO$_2$-SE step 3 | 15 | 353.15 | 0.2542 | 0.0428 | 0.6344 | 0.9154 | 0.21$^b$ ± 0.03 | 143.71$^b$ ± 4.34 | 129.65$^a$ ± 0.99 | 227.78$^a$ ± 2.21 |
| 9   | scCO$_2$+ EtOH$_{H2O}$ | 15 | 353.15 | 0.0488 | 0.0645 | 0.8765 | 0.8369 | 0.70$^f$ ± 0.03 | 124.83$^c$ ± 0.78 | 107.25$^b$ ± 0.54 | 206.54$^b$ ± 0.75 |

$^a$ scCO$_2$ using ethanol with co-solvent (scCO$_2$+EtOH)
Different letters indicate significant differences according Tukey’s test (p < 0.05)
Table 5. Chemical composition of *A. lappa* leaves extracts obtained by the different extraction methods employed

| Compound                  | RT (min) | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|---------------------------|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Triethyl phosphate        | 15.23    | 5.99| 6.10| 3.91| 1.38| n.d.| n.d.| 0.61| 1.7 | 0.66|
| 2,4-Di-tert-Butyl Phenol  | 32.27    | 5.96| 7.25| 6.49| n.d.| 5.93| n.d.| 0.39| 0.84| n.d.|
| Heptadecane               | 39.58    | 8.72| 3.37| 6.96| n.d.| 6.84| 8.91| 0.55| 1.30| 0.54|
| Phytol acetate            | 44.09    | n.d.| n.d.| n.d.| 1.32| n.d.| 7.69| 2.13| 4.26| 1.76|
| Phytol                    | 46.75    | 10.45| 10.45| 10.64| 0.98| 4.76| n.d.| 0.88| 1.61| 0.72|
| Docosane                  | 52.75    | 2.55| 4.72| 3.84| n.d.| 3.38| n.d.| n.d.| n.d.| 0.55|
| Cyclohexasiloxane         | 62.60    | n.d.| n.d.| n.d.| n.d.| n.d.| 6.15| 1.46| 1.43| 1.36|
| Diisooctyl phytalate      | 64.58    | 5.13| 2.88| 6.32| 0.48| n.d.| 6.42| 19.89| 38.60| 0.97|
| Tetracontane              | 72.77    | 2.56| 3.40| n.d.| 2.28| 3.51| 6.06| 0.43| n.d.| 2.56|
| Hexatricontane            | 77.05    | 2.19| n.d.| n.d.| n.d.| n.d.| 1.73| 1.34| n.d.| 9.33|
| Y-sisterol                | 81.93    | n.d.| n.d.| n.d.| n.d.| n.d.| n.d.| 1.14| 2.22| 1.46|
| Lupeol acetate            | 85.03    | 11.49| 13.76| 13.58| 1.65| n.d.| n.d.| 0.97| n.d.| n.d.|
| α-amyrim                  | 85.88    | 4.19| n.d.| n.d.| 6.18| 3.81| 2.44| 7.87| 4.49| 9.83|
| Amyrin acetate            | 87.5     | 5.64| n.d.| 12.57| 6.94| 5.71| 18.23| 6.32| 13.80| n.d.|
| Lupeol                    | 91.10    | 13.09| 4.82| 6.66| 20.28| 17.65| 8.59| 34.52| 19.52| 36.24|

RT: retention time; n.d.: not detected. Extracts: (1) PLE, 15 MPa / 313.15 K; (2) PLE, 25 MPa / 313.15 K; (3) PLE, 15 MPa / 353.15 K; (4) PLE, 25 MPa / 353.15 K; (5) PLE, 20 MPa / 333.15 K; (6) scCO₂ SE step 1; (7) scCO₂ SE step 2; (8) scCO₂ SE step 3; (9) scCO₂ + Aq. EtOH.