EXTRACTS FROM PITANGA LEAVES (*Eugenia uniflora* L.) WITH SEQUENTIAL EXTRACTION IN FIXED BED USING SUPERCRITICAL CO_2, ETHANOL AND WATER AS SOLVENTS

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Abstract. Eugenia uniflora L, also known as Pitanga tree, is a tropical tree very common in South American countries. Its leaves are responsible for many therapeutic activities, and have been used by folk medicine at fever treating, stomach diseases, hypertension, bronchitis and cardiovascular diseases. They have sedative, anti-inflammatory and diuretic actions, and have also antioxidant activity. To obtain the natural extracts containing the bioactive substances responsible for these activities, the extraction processes are been developed searching for better quality products, free of solvents trace and concerned about environmental risks. Supercritical carbon dioxide as a substitute for some organic solvents and water steam is considered an excellent option for these processes. The objective of this study is to obtain different extracts from leaves of Eugenia uniflora L. with sequential extraction technique and compare the results with conventional extraction methods and fixed bed methods, evaluating its extraction performance on the yield and on the concentration of phenolics and total flavonoid content. The sequential extraction in fixed bed used supercritical carbon dioxide, ethanol and water as solvents (60°C and 400 bar). The results have shown that the combination of processes was advantageous whether compared to the respective aqueous or ethanolic extractions, it was also advantageous of producing fractionated extracts. The extraction yields were higher and the obtained extracts by supercritical fluid extraction were more concentrated in active polar compounds. The effect of a prior extraction with supercritical CO_2 produced extracts with high content of phenolic compounds.

Keywords: *Eugenia uniflora L.*, supercritical extraction, phenolics, flavonoids.

1. Introduction

Currently there is great interest in formulations that incorporate extracts from natural sources, to yield products in form of functional foods, cosmetics or medicinal products which contain biologically active substances [1]. Natural extracts are liquid preparations, semi-solid substances or solid substances obtained from biological matrices. These extracts are obtained by contacting the vegetable/ animal matrix with different solvents, such as water or organic solvents. For production of essential oils, process as steam distillation and hydrodistillation has also been employed.

The search for better quality products, free of traces of solvents, as the concern about environmental risks, has led to the search for alternative methods of extraction, cleaner and sustainable. Among these processes stands out the supercritical fluid extraction, using the gas carbon dioxide.

Separation processes employing supercritical carbon dioxide ($scCO_2$) as a substitute for some organic solvents and steam (hydrodistillation or steam distillation) present as a highlighted options to obtain natural extracts containing bioactive substances. Among the advantages of using supercritical carbon dioxide in extraction processes, there is the fact that carbon dioxide possesses low cost, is non-toxic, non-flammable, inert and has good capacity to extract due to its penetration [2]. This technology is successful in cases of

bioactive compounds extraction (antioxidants, essential oils, carotenoids, phenolic compounds, flavonoid, among others.) in a wide variety of plants.

In the last two decades there has been a growing interest in bioavailability and biological effects of flavonoids and phenolic compounds present in various kinds of plants [3]. Among these stands out *Eugenia uniflora* L. (local name in Brazil: pitanga).

Considering the popular use of the leaves of *E. uniflora* L. as anti-hypertensive, studies have reported the presence of flavonoids in its essential oil. Thus, a wide variety of solvents has been used to obtain extracts of pitanga leaves such as methanol, ethanol, steam, supercritical CO_2 and water, isolating and identifying various major components.

Furthermore, studies have shown that phenolic compounds have significant effects on the reduction of cancer. Epidemiologic studies show an inverse correlation between cardiovascular disease and consumption of foods rich in flavonoids, possibly by its antioxidant properties.

The production of concentrated bioactive compounds requires a lot of extraction techniques, especially the purification, due to strict specification of the common products in the food, pharmaceutical and cosmetic sectors, requiring frequent changes in production techniques and purification, always aiming to get economic products and meeting the requirements of current quality [4].

The objective of this work is to obtain different extracts of pitanga leaves by sequential extraction technique, assessing the advantages and disadvantages of this technique, and compare the results of extraction with ethanol extraction methods and conventional aqueous and fixed bed.

2. Materials and Methods

2.1 Raw material characterization

Pitanga leaves samples (*E. uniflora*) were collected and dried (in oven with forced air at 42 ºC for 3 days) from experimental field at the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQB, Campinas, Brazil).

The dried leaves were milled in a knife mill (Marconi, model MA-340, Brazil), classified according its size by sieves in a vibratory sieve system (Bertel, model 1868, SP, Brazil) for 15 minutes. After that, the samples were packed in plastic bags and stored in a domestic freezer (BVR28GBANA, Brastemp, Brazil) at -26 °C.

The sample was characterized as the total content of volatile + moisture (% VM) by gravimetric method AOAC 930.04 [5], the moisture (% M) contend determined by Karl Fisher method (Methom 701 KF Tritino equipped with 832 KF Thermoprep), average particle diameter by ASAE method [6] and real density (ρ_r) determined by helium gas pycnometry (Micromeritics, Accu Pyr II 1340 V1.02).

The particle bed was evaluated for their apparent density (ρ_a) by the method described by Uquiche et al [7] and the bed porosity (ε) was calculated from the real density (ρ_r) of the sample and the apparent density (ρ_a).

2.2 Methodology for obtaining the extracts

The plant matrix was subjected to extraction processes in fixed bed extractor in three steps, using three different solvents: supercritical CO_2 (first), ethanol and water, (second and third, respectively) according Figure 1. For comparison, experiments were performed in a fixed bed with ethanol and water in single stage, without prior extraction with supercritical CO_2, (Figure 2). To complement the results, conventional extractions were performed (low pressure, using ethanol and water as solvents).

Sequential extraction. The experimental apparatus used for the sequential extraction is show in Figure 3. It consisted of a CO_2 supply tank (1), bath of refrigeration (2), high-pressure pump (3), CO_2 tank (4), extractor (5), glass flask collector (7), gas flow meter (9) volume totalizer (10), a heater (11), the trap prepared with Porapak-Q (80/100 mesh, Waters Corporation, Milford, MA) (8) when there is the need to capture volatile fraction and peristaltic pump (6), used to inject solvent and for wash the process line.

In all cases the extractor (5) was bundled manually with approximately 7.0 g dry and ground material. Operating conditions were set at 400 bar and 60 °C for experiments. When the requirements were met, a period of 1/2 hour was adopted as time to stabilize and began leaking extraction by 1.5 L/min of CO_2 through the bed, picking up the bottle collector extract (7), which was weighed on an analytical balance (BEL, model U210A, SP, Brazil). The gaseous CO_2 leaving the collector (7) was drained through a trap Porapak-Q (8) and

led to a flow meter (9) and totalizer (10) for measuring the carbon dioxide used. All tests have been carried out in triplicate.

Fixed bed aqueous and ethanol extraction. The experimental system for aqueous or ethanolic extraction in fixed bed and single step is shown in Figure 4. For this stage, an experimental procedure similar to the above has been adopted (Figure 3). The extractor was packed with 7.0 g of sample and ethanol or water was drained from the bed using a pump of co-solvent (12).

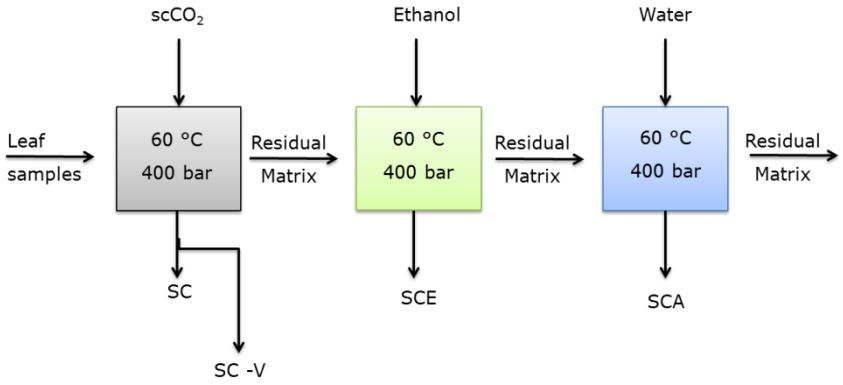

Figure 1. Extracts obtained from the sequential extraction process in fixed bed in three steps. First step: supercritical extract (SC) and volatile supercritical extract (SC-V), second stage: ethanol extract after supercritical extraction (SCE) and the third phase: aqueous extract after supercritical extraction (SCA).

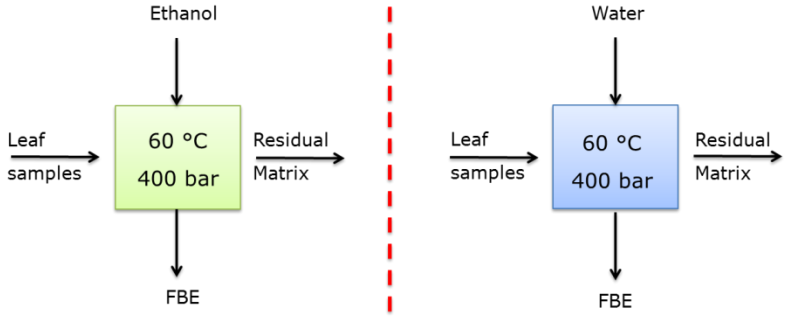

Figure 2. Extracts obtained from the extraction processes in fixed bed in one step. In ethanolic extraction: fixed bed ethanolic extract (FBE) and aqueous extraction: fixed bed aqueous extract (FBE).

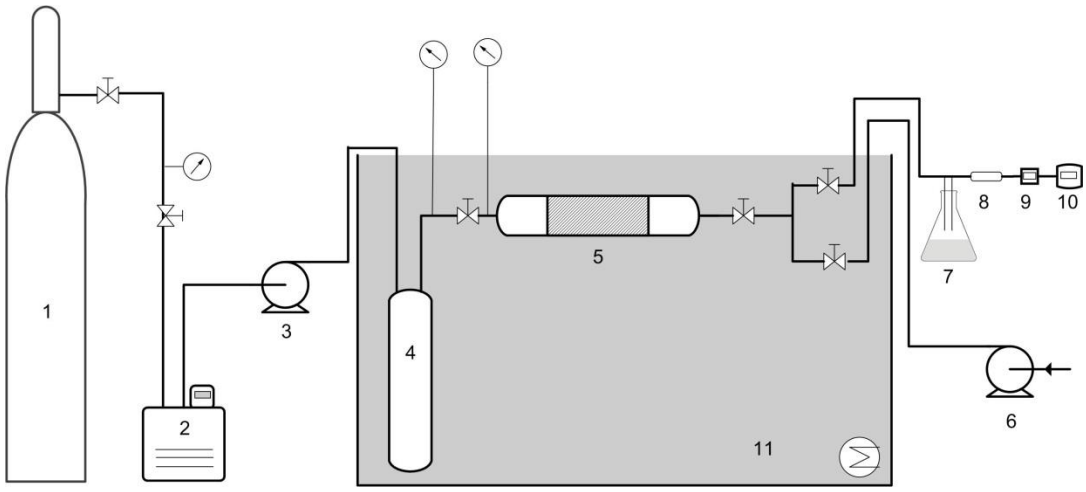

Figure 3. Supercritical CO_2 extraction apparatus.

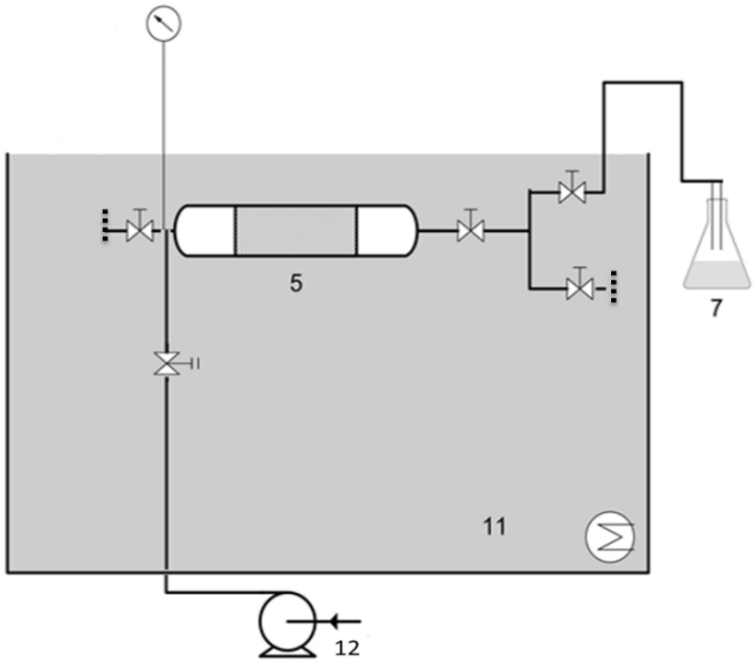

Figure 4. Fixed bed extraction ethanolic or aqueous apparatus.

Extraction conventional aqueous and ethanolic. The extracts were obtained according to the modified methodology of Piantino et al. [8]. Approximately 5.0 grams of dry sample were mixed with 50 ml of solvent (water or ethanol) in an equilibrium cell where the mixture was stirred with the help of a magnetic stirrer and the temperature controlled by a water bath (60 °C) connected to the cell. A reflux condenser (5 °C) was added to the system, whose purpose is to prevent the loss of desirable compounds through evaporation during extraction. The mixture remained stirring for 2 hours and then was vacuum filtered. The filtrate was set aside, and the residual matrix passed again by the extraction step, repeating the process two more times. Each new filtrate was mixed with the former, forming the extract. The solvent present in the ethanolic extracts were evaporated at 50 °C in a rotary evaporator (Marconi, MA120, Brazil). The water present in the extracts was lyophilized (Liobras, model L101, SP, Brazil) to obtain the dry extract.

2.3 Determination of the global extraction yields

As comparative parameter between the different extraction methods, the global extraction yield were employed, which expresses the relation between the dry extract mass obtained in extraction processes and the sample mass used in the process. This yield is based per unit of raw material mass used. The experiments were performed in triplicate, so the overall yield is the result of the arithmetic mean of the experimental values.

For ethanolic and aqueous supercritical extractions in fixed bed, the the global extraction yield was calculated as the relation between the total mass of extract and initial sample mass. In these extractions, yield was also calculated throughout the process, from the extracts dried pasta at fixed time intervals (kinetic).

2.4 Determination of total Flavonoids and Phenols

Total flavonoids were measured as described by Zhishen et al. [9]. The absorbance was measured at 510 nm, using a UV/VIS spectrophotometer (UV–VIS lambda 40, Perkin Elmer, USA). The extracts and calibration curve determinations were carried out with three replicates, and the results were expressed in catechin equivalent (mg CE/g dry extract).

The determination of total polyphenols was performed using the Folin-Ciocalteu second procedure Singleton et al. [10] and expressed as gallic acid equivalents (GAE/g). The absorbance was measured at 750 nm.

3. Results and Discussion

3.1 Raw material characterization

Table 1 shows the properties that characterize the samples *Eugenia uniflora* L.

Table 1. Results of the characterization of raw material.

Characterization	Properties	Results
Sample	VM (%)	7,17 ± 0,15
	M (%)	5,90 ± 0,08
Particle bed	ρ_r (g/cm³)	1,50 ± 0,01
	d_{mg} (mm)	0,336 ± 0,003
	ρ_a (g/cm³)	0,456 ± 0,009
	ε	0,696 ± 0,02

Results presented in Table 1 show the 1.3% difference between the moisture values determined by Karl Fisher (M %) and the value obtained by gravimetric determination (% VM). In the gravimetric method, there is the evaporation of volatiles with water, mistakenly counting the mass evaporated as moisture.

3.2 Extraction yields and kinetic extraction

Figure 5 compares the global extraction yields obtained by extraction with $scCO_2$, (SC) ethanolic extraction after supercritical extraction (SCE), aqueous extraction preceded by supercritical and ethanol extraction (SCA), ethanolic (FBE) and aqueous (FBA) fixed bed extraction, low pressure extraction with water (A) and ethanol (E) and the accumulated global yield of sequential extraction (SC + SCE + SCA). The results were calculated from averages of experiments conducted in triplicate.

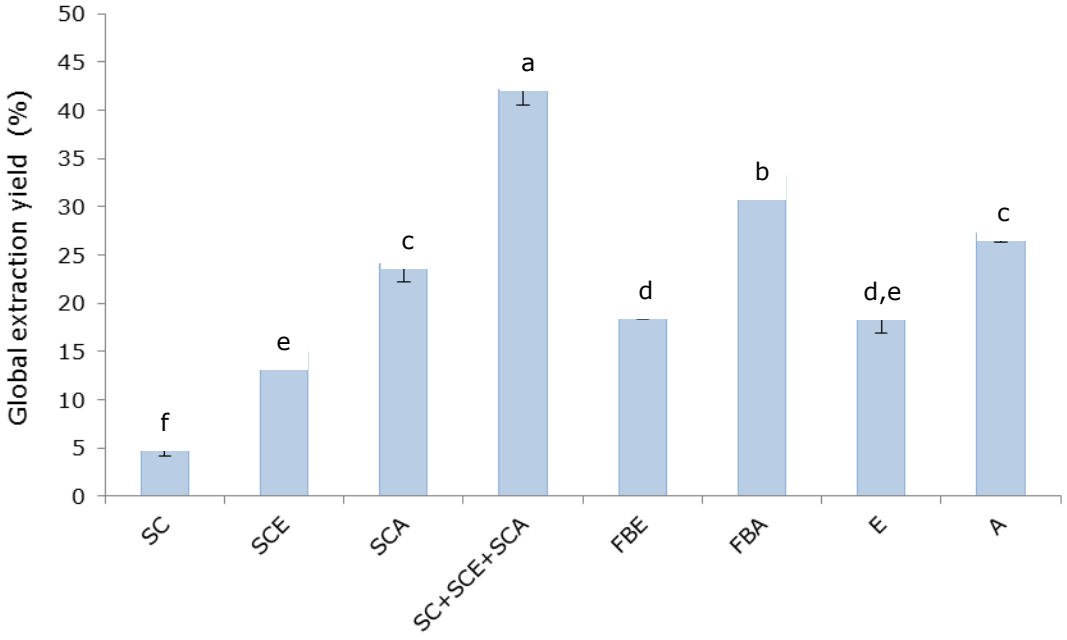

Figure 5. Average values of yields of extracts obtained from *E. uniflora* by supercritical extraction methods (SC), ethanolic post supercritical extraction (SCE), aqueous after ethanol and supercritical extraction (SCA), fixed bed extraction ethanolic (FBE) and aqueous (FBA), low pressure extraction with water (A) and ethanol (E) and the accumulated global yield of sequential extraction (SC + SCE + SCA). The bars represent the mean value ± SD. Different letters represent statistically significant differences (Tukey test at 5% significance level).

For *E. uniflora*, the character of the solvent used greatly influenced the process of obtaining extractables, as yields showed a tendency to increase with increasing solvent polarity: aqueous extract > ethanol extract > $scCO_2$ extract.

The preliminary extraction with supercritical CO_2 can promote changes in the interactions between compounds and residual matrix hindering or facilitating its subsequent extraction [11,12]. Furthermore, SCO_2 also promotes a change in the structure of the solid matrix due to the use of high pressure and subsequent depressurization. [13]. In the case of the pitanga leaves, the supercritical extraction showed no significant influence between the yields of ethanol and aqueous extracts. It was noted that the yields of conventional extracts are statistically equal to those of extracts preceded by supercritical extraction.

The results obtained in the different processes show a significant difference between the supercritical, aqueous and ethanolic extraction yields, a fact explained by both the selectivity and polarity of the solvent and the nature of the compounds present in the plant matrix.

In general, the aqueous extractions (SCA, FBA and A), had the highest yields, followed by ethanolic extraction and lastly the supercritical extraction.

Sequential and single phase extraction curves obtained at 400 bar and 60 °C using 1.5 L / min of $scCO_2$ and 0.5 ml/min of ethanol and water are shown in Figures 6 and 7. These curves show the accumulated yield of extract based on the mass of solvent used in extraction by sample mass, indicating the ease or difficulty with which the solutes are extracted. Given that the extraction is influenced by the solubility of solutes in solvents, particle size and diffusivity of solute and solvent in the solid matrix, the nature of the solutes and the particle size are critical for these curves.

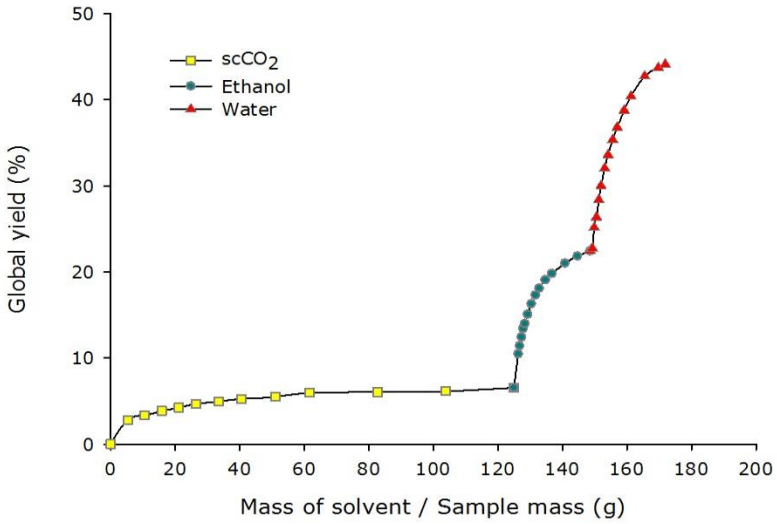

Figure 6. Accumulated global yield and kinetics of sequential extraction with supercritical CO_2 ($scCO_2$), ethanol and water from *Eugenia uniflora* L.

The first stage of the process of supercritical extraction (Figure 6), show a high removal rate of extract. After this phase, the curve seems to quickly reach a constant value, which would be an indication that the plant matrix was almost exhausted of soluble in $scCO_2$. The kinetics of the extraction process indicates that the compounds are easily extractable soluble and have low resistance to mass transfer.

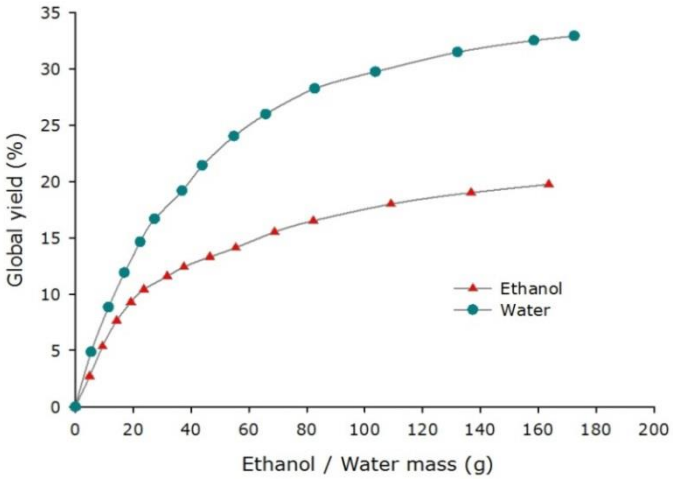

Figure 7. Global yield and extraction kinetics in fixed bed with ethanol and water from *Eugenia uniflora* L.

At ethanolic and aqueous extraction steps, yield increased gradually at an increasing rate in the early extraction and did not reach a steady rate in the final points of the curve, which would indicate that the plant matrix was not fully depleted during the process.

3.3 Total polyphenol (TPC) and Total flavonoid (TF) contents

The Figure 8 show the data concentration of phenolic compounds in extracts of *E. uniflora*.

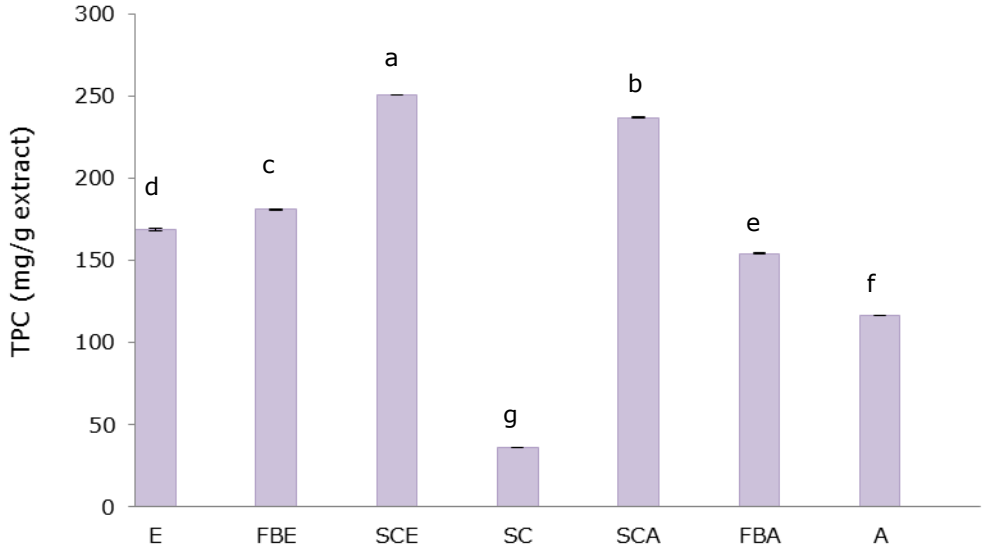

Figure 8. Total polyphenol (TPC) in the extracts of *E. uniflora*. The bars represent the mean value ± SD of triplicate assays. Different letters represent statistically significant differences (Tukey test at 5% significance level). Supercritical (SC), ethanolic (SCE) and aqueous (SCA) sequential extract, ethanolic (E) and aqueous (A) conventional extract (low pressure), ethanolic (FBE) and aqueous (FBA) in fixed bed (high pressure).

The content of phenolic compounds in the extracts, both in single-step processes and in sequential extraction involving ethanol, were well above the contents with other solvents under the same conditions.

The previous extraction with supercritical CO_2 significantly improved the yield of polyphenolic compounds. These results suggest the use of supercritical extraction followed by ethanol and aqueous extraction to obtain extracts with higher content of total polyphenol compounds.

Figure 9. Total flavonoid (TF) contents in the extracts of *E. uniflora*. The bars represent the mean value ± SD of triplicate assays. Different letters represent statistically significant differences (Tukey test at 5% significance level). Supercritical (SC), ethanolic (SCE) and aqueous (SCA) sequential extract, ethanolic (E) and aqueous (A) conventional extract (low pressure), ethanolic (FBE) and aqueous (FBA) in fixed bed (high pressure).

Figure 9 compares the total flavonoid (TF) contents of the extracts obtained by different extraction methods.

In the first step of the sequential extraction (SC) there is a high content of flavonoids, which can be explained in terms of low polarity phenolic compounds extractable $scCO_2$.

It is also observed that in the case of aqueous extraction preceded by $scCO_2$, the concentration of flavonoids is approximately equal to the concentration of the extracts obtained in one step, not happening the same when ethanol is used.

4. Conclusions

This study showed that the sequential extraction process obtains extracts with different compositions and features.

The supercritical extraction before the ethanolic and aqueous extraction had positive influence, producing extracts with higher content of phenolic compounds.

The maximum extraction of phenols was obtained by ethanolic extraction preceded by the high pressure supercritical at the same conditions.

This work represents part of the dissertation´s results that will be defended in August 2013.

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