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A novel cascade approach to extract bioactive compounds from officinal herbs

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PAPER

Abstract

This research aims to compare a novel cascade extraction method with a conventional solid–liquid extraction method, both assisted by ultrasounds. The cascade extraction method consists of a sequential series of extractions performed with the same hydroalcoholic solvent, which is reused from one herb to the other. In practice, a hydroalcoholic solution is firstly used to extract one botanical herb. The resulting extract is then reused for the extraction of a second herb. The process is repeated as many times as the number of herbs composing the final formulation. The main advantage of this approach is firstly the lower need of solvents compared with the individual extraction procedures, where a fresh solvent is needed on each extraction step. Furthermore, extracts of the two methods (solid liquid vs cascade extraction) were characterized with several antioxidant assays (DPPH, ORAC, and FRAP) and total phenolic content (TPC). The results show that the solid–liquid extraction method achieves similar yields to total phenols and similar TAC in comparison to the extracts obtained by the cascade extraction method. Also, the HPLC analysis of the extracts showed that both methods lead to similar chromatographic profiles either when analyzed by an electrochemical detector (CoulArray) or by a spectrometric diode array detector (DAD). However, our findings support the idea that the cascade extraction method obtains extracts richer of minor peaks, showing a more complex bioactive profile. Such results could be explained considering that the solvent used during the series of cascade extractions was enriched not only by antioxidants but also by plant surfactants, like saponins, which increase the solvent solubility. Overall, this research shows that the cascade extraction method can not only provide officinal herb extracts with similar phenolic yield and antioxidant capacity than conventional solid–liquid extraction but also with a more complex bioactive profile compared to traditional solid–liquid extraction and with a minor consumption of solvents.

Keywords: antioxidant ability; cascade extraction; herbal tincture; HPLC

Introduction

Herbal extracts are hydroalcoholic preparations typically obtained by maceration or percolation (Karioti *et al.*, 2011). After extraction with a hydroalcoholic solution, the resulting extracts contain a complex profile of bioactives that can be used as supplements for human health. Several studies report the beneficial effect of extracts obtained from botanicals. For example, according to Dos Reis *et al.*, the extract of *Ptychopetalum olacoides*, which contains tannins, flavonoids, and terpenoids, is used as a remedy for problems concerning the central nervous system (Dos Reis and Mendes, 2018). Also, the extract prepared with *Turnera diffusa* has anxiolytic and antidepressant effects (Ana María *et al.*, 2019). However, the composition in bioactives depends on a proper extraction technique.

The most traditional way to prepare extracts is the classical maceration of dry herbs with a hydroalcoholic

solution, in a solid-to-liquid ratio of 1:–5 or 1:–10. With slight modifications, other parent techniques have been developed, like percolation, hydro-distillation, boiling with reflux, and Soxhlet (Alara *et al*., 2018). Unfortunately, such techniques consume large amounts of organic solvents with high purity, and have limited extraction yields and low selectivity. Moreover, they need long extraction times, which may cause the thermal degradation of some sensitive bioactives (Rostagno *et al*., 2010).

Recently, the efficiency of the process has been greatly improved by implementing innovative technologies, such as ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), and microwave-assisted extraction (MAE) (Reverchon and De Marco, 2006; Valachovic *et al*., 2001; Xia *et al*., 2011). When traditional extraction is assisted with such techniques, the duration of the extraction process is greatly reduced (i.e., a few hours), maximizing the yield of the extracted bioactives and limiting thermal degradation problems (Valachovic *et al*., 2001; Vinatoru *et al*., 2017).

Despite such improvements, the extraction process still has some limitations. The most evident is that many lipophilic bioactives present in herbs (like carotenoids or terpenes) are scarcely soluble in hydroalcoholic solvents. This clearly limits the potential quality, potency, and effectiveness of the resulting extracts. For this purpose, some recent researches have tried to overcome the low solvation capacity of pure hydroalcoholic solvents by the addition of ionic liquids (Han *et al*., 2011), lime juice (Cheok *et al*., 2013), diethylamines (Choi *et al*., 2000), and saponins. Through the careful use of some additives, important solvent properties like viscosity, pH, or polarity can be fine-tuned as required. This, ultimately, could increase the extraction yield of bioactive compounds.

A further important limitation on the current use of traditional extraction techniques is the high solvent consumption. Generally, for each volume of dry herbs, 5–10 volumes of fresh hydroalcoholic solvent are needed (Council of Europe, 2019). Although such high solvent to sample ratio is intended to maximize the yield of extractable compounds, it represents a relevant manufacturing cost, a high energy demand, and an environmental concern (Abubakar *et al*., 2015).

Accordingly, this work aims to propose a simple but ingenious cascade extraction protocol, which reuses the same initial hydroalcoholic solvent in a series of extraction cycles. In practice, the procedure starts with a hydroalcoholic solvent, which is used for the first extraction of one selected botanical herb. Then, the resulting extract is recycled for the extraction of a second selected botanical herb. The procedure continues in a series of extractions, where the extract of each step becomes the solvent for the subsequent step. Thanks to such an innovative extraction procedure, during the reutilizations of the solvent, and through the wise selection of the herbal sequences, the hydroalcoholic solvent is progressively enriched not only with the main characteristic bioactives from each botanical herb but also with natural modifiers, which fine-tune the viscosity, pH, and polarity of the initial hydroalcoholic solvent. This, ultimately, will enhance its solvation capacity, leading to final extracts with richer bioactives profile, higher yields, and less solvent consumption.

Thus, in this work, the extraction efficiency of a traditional solid–liquid extraction method is compared with the proposed cascade extraction approach. Total phenolic content (TPC) together with total antioxidant capacity (DPPH, FRAP, and ORAC) of both approaches were compared. High performance liquid chromatography (HPLC) coupled with diode array detector (DAD) and coulometric array detector (CoulArray) were used to analyze the bioactive compounds extracted with both approaches.

Material and Methods

Reagents

Ethanol, Folin–Ciocalteau reagent, sodium carbonate, gallic acid, 2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), sodium acetate, acetic acid, ferric chloride, hydrochloric acid, disodium phosphate, monosodium phosphate, sodium hydroxide, fluorescein, 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), HPLC grade ammonium formate, formic acid, and acetonitrile were obtained from Sigma-Aldrich (USA). 2,4,6-Tris(2-pyridyl)-s-triazine (98%) was purchased from Alfa Aesar (USA). The Milli-Q water was purified by Sartorius arium® mini (Germany).

Samples

Four varieties of officinal herbs, namely, *Ptychopetalum Olacoides Benth. (stem)*, *Turnera Aphrodisiaca Willd. (leaf)*, *Schisandra Chinensis baill. (fruit)*, and *Poligonum Multiflorum Thunb. (root),* were kindly provided by Naturalsalus® and preprocessed based on their standardized protocols. In brief, the dried herbs were grounded with a laboratory mill (PerkinElmer, Laboratory Mill 3100, Germany) and sieved (Retsch, AS 200, Germen) to reach a uniform size (<250 μm). The powder was then preserved at ambient temperature before extraction.

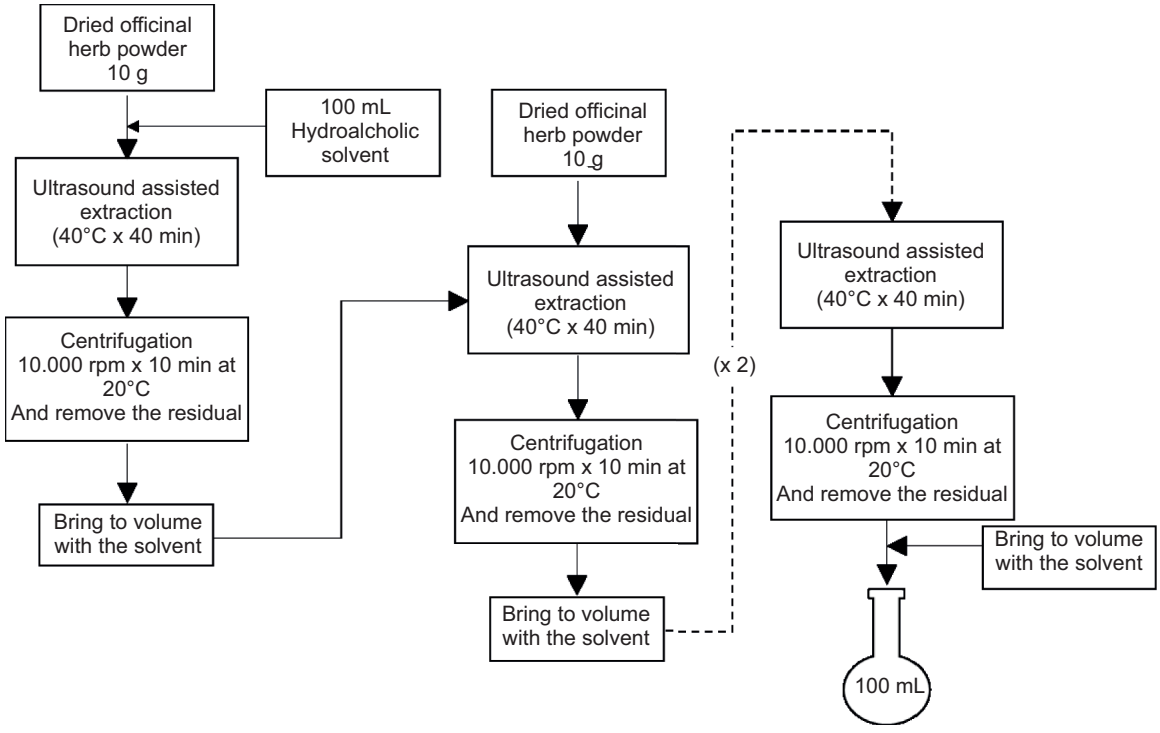

Figure 1. The process of cascade extraction used in the current research.

Extraction

Extractions were performed with an ultrasound reactor (STEEL[®], digital ultrasonic generator, Italy). Briefly, 10 g of dry herbal powder was mixed with 100 mL of solvent (water: ethanol = 1:1, *v/v*) and extracted for 40 min at 40°C. Ultrasound power was set to 60% (345 W, 38 kHz). After extraction, the resulting solution was filtered (PTFE 0.45 µm, Pall Corporation, USA) and centrifuged (10,000 rpm for 10 min at 20°C, SL 16R Centrifuge, Thermo Scientific, USA). The resultant supernatant was collected and stored at –80°C.

Cascade extraction consists of using the extract (obtained from an extraction step) as the solvent for the next step. During each step, fresh hydroalcoholic solvent (typically less than 10% of the initial volume) could be added to replace the solvent evaporated and trapped in the herb matrix during the operation, and maintain a fixed initial sample to solvent ratio (1:10). The experimental conditions of solid–liquid ratio, extraction time, temperature, and ultrasound power were applied equally to each extraction step modified from the previous officinal herb extraction conducted by Rodríguez-Pérez *et al*. in 2015 (see Figure 1). The optimized sequence used in this work is the following: *Ptychopetalum*, *Turnera*, *Schisandra*, and *Poligonum*. For comparison, individual extraction was also performed, similar to cascade extraction, which compensated for the lost solution (100 mL) before performing the analysis.

Total phenol content

The TPC analysis was based on Margraf *et al.* with small modifications (Margraf *et al*., 2015). Briefly, 110 µL of distilled water, 10 µL of sample or gallic acid standard, 10 µL of Folin–Ciocalteu reagent, and 10 µL of saturated sodium carbonate solution were added to a 96-well microplate. The microplate was incubated for 120 min at 25°C. Finally, the absorbance of each well was measured at λ = 765 nm (Tecan, Infinite M Nano⁺, Switzerland). From the absorbance measurements, the total phenol content was expressed as gallic acid equivalent (GAE) based on a calibration curve, built with a series of gallic acid standards.

Antioxidant assays

DPPH assay

The free radical scavenging activity was measured with 2,2-diphenyl-1-picrylhydrazyl stable radical (DPPH¨). 160 µL of DPPH working solution (125 µM) was added to 40 µL of Trolox standards or herbal extracts into a 96-well microplate. The mixture was left to stand for 30 min in the dark at room temperature. The absorbance was measured at 515 nm and the result was expressed based on the Trolox equivalent (Mishra *et al*., 2012).

FRAP assay

The ferric reducing antioxidant power (FRAP) assay was conducted based on previous research by Benzie

and Strain with modifications (Benzie and Strain, 1996). 180 µL of FRAP reagent (pH = 3.6 acetate buffer, 20 mM ferric chloride, 40 mM TPTZ in 40 mM hydrochloric acid in 10:1:1 ratio) was added to 20 µL of Trolox standard or herbal extracts. After incubation for 1 h at 25°C, the absorbance at 593 nm was recorded and the result was expressed as µmol TE/g DW.

ORAC assay

The oxygen radical absorbance capacity (ORAC) assay was performed using the method modified from Prior *et al.* (2003). Phosphate buffer (75 mM, pH 7.0) was used to dilute all the solutions, including fluorescein (3.19 µM), AAPH solution (111 mM), Trolox, and herbal extracts. 30 µL of sample or Trolox standard was premixed with 75 µL of fluorescein and incubated at 37°C for 5 min before adding freshly prepared 75 µL of AAPH in a 96-well microplate. The fluorescence signal was monitored every minute, during 2 h reaction time with excitation wavelength at 485 nm and emission wavelength at 535 nm. The antioxidant capacity was calculated based on the area under the fluorescence decay curve and expressed as µmol TE/g DW.

HPLC analysis

The sample extracts were analyzed with Agilent 1260 Infinity HPLC system with a binary pump, an autosampler with the temperature control system, a DAD (Agilent Technology, USA), and a 16 channel CoulArray detector (ThermoFisher scientific, USA). A Kinetex Biphenyl column (100 × 2.1 mm, 2.6 µm particle size with a precolumn, Phenomenex, USA) was applied for the separation of compounds at constant 30°C. The separation was conducted with 6.5 mM ammonium formate in Milli-Q water with 0.1% formic acid as phase A, and 6.5 mM ammonium formate in acetonitrile with 0.1% formic acid as phase B. 5 µL of the sample was injected with a constant flow rate of 0.3 mL·min^{-1}. All the samples were previously loaded into the autosampler and kept at 5°C before analysis. The elution gradient was 98% of phase A from 0–8 min, then down to 40% at 20 min, to 5% A at 27 min, kept constant for 2 min, and the final equilibration was 98% A at 31 min. The DAD detector was set at 280 nm and the CoulArray was set from 50 to 800 mV, with 50mV increment through the 16 electrodes (vs. Pd electrode).

Results

The total phenolic content

Table 1 presents the TOC of the extracts obtained by using the traditional maceration process, either of individual herbs (obtained under the same sample to solvent ratio) or their mix in comparison with the proposed cascade method. Among the individual herbs, the *Poligonum* (10 g in 100 mL of fresh solvent) showed the highest TPC (27.3 ± 3.3 mM), while the *Ptychopetalum* (10 g in 100 mL of fresh solvent) showed the lowest (3.49 ± 0.20 mM). The combination of the herb extracts (each obtained with a sample to solvent ratio of 1:10) resulted in a total phenol content of 59.1 ± 7.3 mM of gallic acid equivalent.

Concerning the extracts obtained with the proposed cascade extraction, the TPC was 57.0 ± 1.7 mM. Such a result is not significantly different from the mix of individual extracts (t-test, $P < 0.05$). Furthermore, it is important to highlight that, although the cascade extraction used the same amount of dry herbs as the traditional maceration method (10 g of each botanical), the volume of hydroalcoholic solvent needed was about one fourth. These findings suggest that the cascade extraction method is able to extract similar amount of phenolic compounds than the maceration method, but with less solvents.

The antioxidant assays

The results of antioxidant assays, including DPPH, FRAP, and ORAC, are presented in Table 1. In general, those individual sample extracts that showed higher TPC also had higher antioxidant capacity. In detail, the *Turnera* and *Poligonum* showed the highest antioxidant capacity in all the antioxidant assays. On the contrary, the *Ptychopetalum* and *Schisandra* had lower antioxidant capacity. What is striking here is that the extracts obtained with the cascade extraction method have ORAC, FRAP, and DPPH assay values that are not significantly different ($P < 0.05$) from those obtained by mixing the individual extracts. Overall, these results confirm those obtained by total phenol content (*vide supra*).

The HPLC-CoulArray analysis

Figure 2 shows the chromatogram obtained by HPLC coupled with an electrochemical array detector (CoulArray) of the extracts. The electrochemical detector is useful for providing quantitative and qualitative information of each compound eluted from the chromatographic column (Haque *et al.*, 2021). First, the analysis of the peak current values obtained at different applied potentials can be used to determine the redox potential of each eluted species. Furthermore, the peak heights obtained at very high oxidative potentials (i.e., +800 mV vs ref. electrode) can also be used to express the total amount of antioxidant present in the sample, i.e., the total antioxidant capacity (Hicks *et al.*, 2017). From Figure 2, the herbs *Poligonum* and *Turnera* showed higher peak heights than *Schisandra* and *Ptychopetalum*.

Table 1. The TPC, DPPH, FRAP, and ORAC results of individual herb sample extracts and those from cascade extraction.

	TPC (mM GAE)	DPPH (mM TE)	FRAP (mM TE)	ORAC (mM TE)
(1) Ptychopetalum	3.49 ± 0.20	0.37 ± 0.08	1.48 ± 0.20	12.01 ± 0.80
(2) Turnera	22.2 ± 3.4	13.44 ± 0.58	11.98 ± 0.86	51.7 ± 1.2
(3) Schisandra	6.02 ± 0.41	3.00 ± 0.78	5.10 ± 0.58	22.2 ± 2.1
(4) Poligonum	27.3 ± 3.3	13.8 ± 1.2	11.43 ± 0.04	36.57 ± 0.51
Mix of individuals[a]	59.1 ± 7.3	30.6 ± 2.6	30.0 ± 1.7	122.5 ± 4.6
Cascade extraction	57.0 ± 1.7	28.5 ± 3.9	30.5 ± 5.0	123.0 ± 1.7

[a]The accumulation of four individual herb sample extracts.

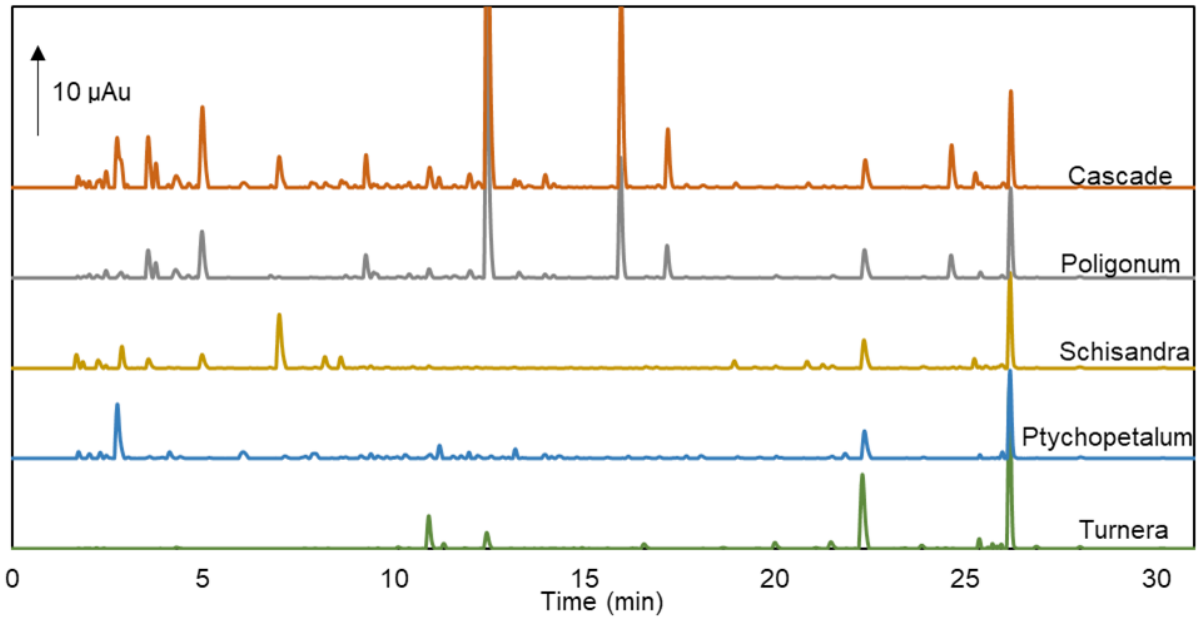

Figure 2. The HPLC-CoulArray result of the individual and cascade sample extracts (potential poised at +800 mV versus Palladium electrode).

This finding correlated with the values of total phenol content and total antioxidant capacity determined previously. Meanwhile, the chromatograms confirmed that the cascade extraction was able to extract all the main redox compounds present in the individual herbs, although with less solvents.

HPLC-DAD analysis

The HPLC-DAD analysis was performed to compare the bioactive compound profile of individual and cascade extraction at 280 nm. Figure 3 shows the chromatograms of all individual herbs and that of the extract obtained by cascade extraction. The results show that the sum of the main peaks observed in the individual herbs is also present in the cascade extraction. This finding confirms the results obtained by the HPLC-CoulArray and supports the conclusion that both extraction methods, i.e., traditional maceration versus cascade extraction, provide extracts with similar composition.

However, through a more detailed analysis of the HPLC-DAD chromatogram, it is possible to observe that the extracts obtained from the cascade method present a more complex profile. With such extracts, several minor compounds (see Figure 4) were observed as small peaks along the chromatographic trace. It is important to highlight that such peaks were not present in the HPLC-DAD of the individual extracts. For instance, the peaks observed at a retention time of around 8.5 min (peaks A, B, and C) and at 13.5 min (peak D) were only present in the extracts obtained by the cascade method, while they were absent in the chromatograms of the individual

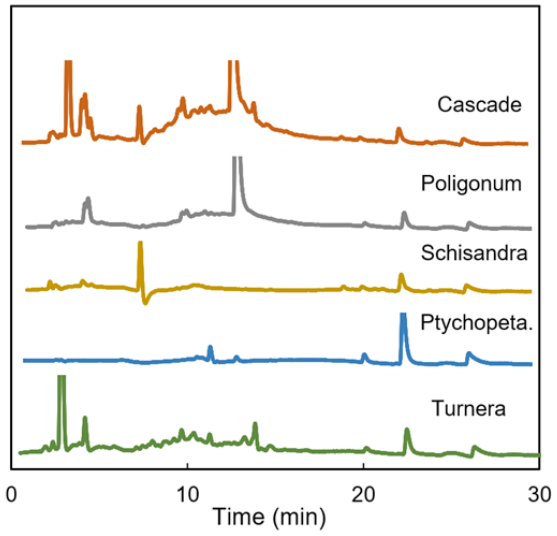

Figure 3. The HPLC-DAD analysis of individual and cascade extraction at 280 nm.

herbs. This finding suggests that the cascade extraction was able to increase the recovery of more minor bioactives than the maceration method of individual herbs.

Meanwhile, Figure 3 shows that the sample extract obtained by the cascade extraction method results in a chromatogram with a lower content of some peaks, if compared with the samples obtained from the single extraction methods. This is clearly observable in the chromatogram of the first herb, *Ptychopetalum*. Such loss of certain phenolic compounds can be explained with their adsorption to the solid retentate, which occurs between one extraction and the other. However, Figure 3 also shows that some other peaks are bigger in the cascade extract than in the individual extracts. This observation may also be explained considering that the cascade extraction generates solvents that are progressively richer not only of phenols but also of natural modifiers, which modify the solvating power of the solvent.

For this purpose, several studies have shown that the botanicals used in this study are naturally rich of surfactants, like saponins, which act as solvent modifiers and could be useful to increase the extraction of certain bioactive compounds (Peng *et al*., 2018; Walthelm *et al*., 2001). In particular, one of the main benefits of using a solvent naturally enriched by saponins is that the solvent viscosity is reduced, the surface tension is lowered, and micelles are generated. Overall, such effects may explain the enhanced solvating power of a solvent enriched by natural saponins (Vinarov *et al*., 2018).

Several extraction methods have been developed focusing on solvent saving during the extraction process. The most traditional and widely applied method is Soxhlet extraction. This method continuously recycles the solvent by a series of evaporation/condensation cycles, while accumulating the extracted bioactives in a boiling flask (Patel *et al*., 2019). In recent years, other continuous and batch extraction techniques have been developed (Poirot *et al*., 2007). Among other methods, accelerated solvent extraction (Richter *et al*., 1996) and Randall extraction (Thiex *et al*., 2003) are very promising for their solvent

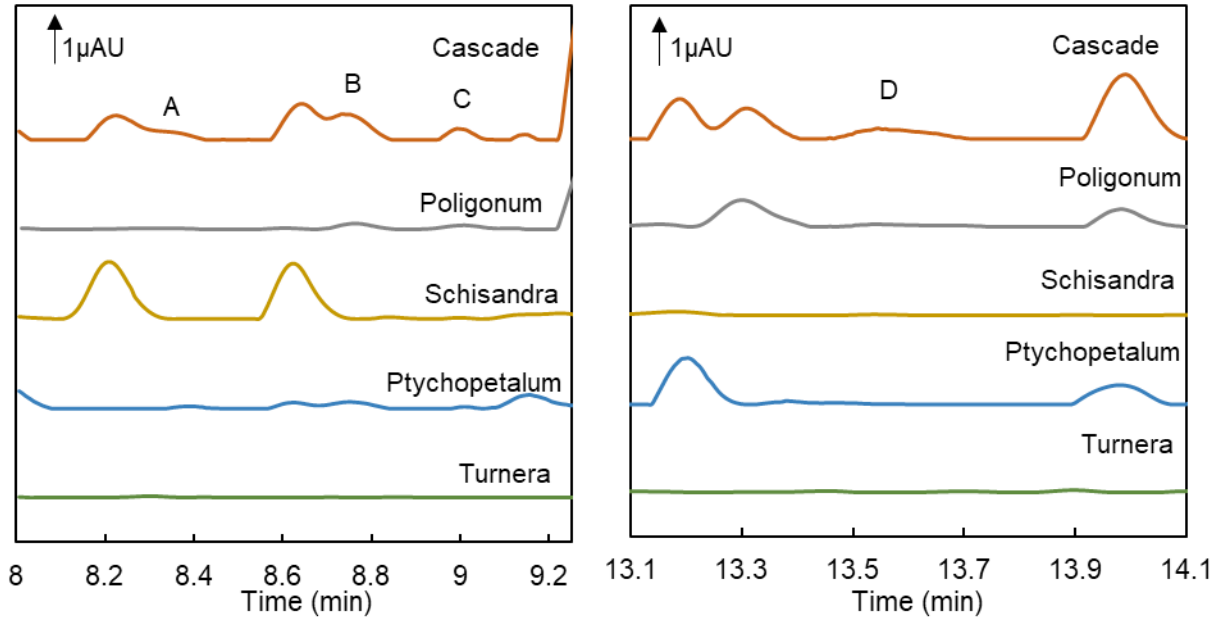

Figure 4. The HPLC-DAD analysis of individual and cascade extraction at certain time 8–9.2 min and 13.1 to 14.1 min.

saving attributes. However, in comparison with the abovementioned extraction technologies, the cascade extraction approach proposed in this research is still more advantageous. For instance, to prepare an extract composed of four individual botanicals, the single extraction method needs a total of 400 mL of solvent for the extraction of 40 g of herbs. On the contrary, the cascade extraction needs only 140 mL of solvent in total. Such solvent reduction is a unique feature of the cascade extraction approach that cannot be simply achieved with other techniques.

Conclusion

This research proposes an innovative extraction method, called cascade extraction, that presents some advantages compared to the traditional solid–liquid extraction (i.e., maceration), especially when the final desired extract is a mixture of several botanical herbs. Although the overall extraction efficiency (determined by TPC, DPPH, FRAP, and ORAC assays, and HPLC analysis coupled with the electrochemical detector) of the proposed cascade extraction procedure is not significantly different from that achieved by the mixture of individual herbal extracts, HPLC-DAD showed that the extracts obtained by the cascade extraction procedure were richer in minor compounds. This means that the resulting extract with the proposed method has a more complex profile of bioactives. Furthermore, it is important to highlight that this result was achieved with less solvents. Indeed, the cascade extraction reuses the same initial solvent during the series of extraction steps, resulting in a simple and more sustainable extract. Moreover, it provides extracts with a richer bioactive profile than the traditional maceration of individual herbs and can be further applied in the food or pharmaceutical industries for the extraction of certain compounds from food or herbal materials.

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