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Comparisons between conventional, ultrasound-assisted and microwave-assisted methods for extraction of anthraquinones from *Heterophyllaea pustulata* Hook f. (Rubiaceae)

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ABSTRACT

This work reports a comparative study about extraction methods used to obtain anthraquinones (AQs) from stems and leaves of *Heterophyllae pustulata* Hook (Rubiáceae). One of the conventional procedures used to extract these metabolites from a vegetable matrix is by successive Soxhlet extractions with solvents of increasing polarity: starting with hexane to eliminate chlorophylls and fatty components, following by benzene and finally ethyl acetate. However, this technique shows a low extraction yield of total AQs, and consumes large quantities of solvent and time. Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) have been investigated as alternative methods to extract these compounds, using the same sequence of solvents. It was found that UAE increases the extraction yield of total AQs and reduces the time and amount of solvent used. Nevertheless, the combination UAE with benzene, plus MAE with ethyl acetate at a constant power of 900 W showed the best results. A higher yield of total AQs was obtained in less time and using the same amount of solvent that UAE. The optimal conditions for this latter procedure were UAE with benzene at 50 °C during 60 min, followed by MAE at 900 W during 15 min using ethyl acetate as extraction solvent.

1. Introduction

The plant kingdom has traditionally been the provider of raw material for the pharmaceutical industry, since it offers a huge range of possibilities for finding new chemical compounds for biological and/or technological applications [1–3]. In the search for biologically active metabolites, the plants traditionally used in folk medicine as well as those recognized as toxic species can be selected, since both groups of plants offer chemical compounds with biological effects potentially useful in therapeutic treatments [4]. Among toxic vegetable species, the subset recognized as phototoxic plants have active principles commonly known as photosensitizers, which trigger their harmful effects under the action of light and [4,5]. Our research group has started the chemical study of the South American vegetal species *Heterophyllaea pustulata* Hook f. (Rubiaceae), popularly known as "cegadera", which has been reported as a phototoxic plant [6,7]. Nine anthraquinones (AQs) with photosensitizing properties, mediated by the generation of superoxide anion ($O_2^{\cdot-}$; type I mechanism) and/or singlet molecular oxygen ($^1O_2$; type II mechanism), were isolated from the aerial parts of the plant [8–10]. Three of these AQs, soranjidiol, rubiadin and 1-methyl ether rubiadin, stand out as the main components of leaves and stems [8]. These AQs have demonstrated important antibacterial and anticancer activity by means of the photosensitization phenomenon [11,12]. In addition, we have previously established that extracts containing these compounds exhibited a significant antibacterial, antifungal and antiviral activity without the photosensitizing effect [8,13]. These records demonstrate the importance of finding a suitable process to extract these AQs from *H. pustulata*.

Traditionally, these AQs have been obtained from the vegetable matrix by performing successive extractions in a Soxhlet apparatus with solvents of increasing polarity, starting with hexane to defeat the vegetal material, following with benzene and finally ethyl acetate [8]. Although this extraction method is faster, less laborious,

Abbreviations: AQs, anthraquinones; UAE, Ultrasound-assisted extraction; MAE, Emicrowave-assisted extraction; $O_2^{\cdot-}$, superoxide anion; $^1O_2$, singlet molecular oxygen; HPLC, High-performance liquid chromatography; t_R, retention times; AUP, area under each peak.

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and consumes less amount of solvent than other conventional methods (maceration, reflux, decoction, infusion, etc.), it presents low selectivity for certain secondary metabolites, such as AQs from *H. pustulata*. Therefore, it is important to develop a more selective and efficient technique to obtain pure anthraquinone derivatives in larger amounts. In this direction, Ultrasonic-assisted extraction (UAE) has been used for the extraction of plant components. With this technique, high extraction yields of good quality have been obtained in shorter periods of time and using lower amounts of solvent than traditional processes [14]. UAE uses high power, and low frequency sound waves to detach the solute of interest from the vegetable matrix. The sound waves that propagate into the solvent media result in alternating high/low pressure cycles, which produces cavitational bubbles. The energy generated from collapsing cavitational bubbles provides greater penetration of the solvent into the cellular material and improves mass transfer to and from interfaces [15]. Among the new extraction techniques, UAE is the most economical and the one with less instrumental requirements [16]. Different plant extracts and bioactive metabolites have been obtained with this technique [17,18]; among them, AQs were isolated from *Morinda citrifolia* [19].

Another alternative technique is microwave assisted extraction (MAE), which has been increasingly investigated in the extraction and isolation of phytocompounds. MAE is governed by two phenomena: ionic conduction and dipole rotation, which in most cases occur simultaneously in polar materials and solvents [20,21]. This makes the temperature of the solvent to increase, enhancing solute solubility [22]. Thus, it has been found that MAE can lead to a considerable increase in the yield of AQs extraction [23,24].

In this work UAE and MAE techniques have been applied for extracting soranjidiol, rubiadin and 1-methyl ether rubiadin from the aerial parts of *H. pustulata*. For both techniques, the effect of different conditions (time, power and/or temperature) over the quality and quantity of the AQs extracted from leaves and stems was assessed, in the search of a faster technique that uses fewer amounts of solvent, time and energy. These results were compared with those obtained with the conventional Soxhlet extraction method.

2. Materials and methods

2.1. Plant material

Aerial parts of *H. pustulata* were collected in La Almona, Jujuy province, Argentina, in January 2011. The material was identified by Prof. Dr. Gloria Bardoza (Instituto Multidisciplinario de Biología Vegetal, IMBIV-CONICET), and a voucher specimen has been deposited at the Cordoba Botanical Museum as CORD 305.

2.2. Solvents

The solvents used in the extractions were: n-Hexane (Biopacks, 98.9%), benzene (Taurus, 96.7%) and ethyl acetate (Sintorgan, 99.5%).

2.3. Conventional Soxhlet extraction

Air-dried aerial plant material was separated into stems and leaves. Five grams of stems and 3 g of leaves were mechanically triturated and treated with 180 mL of hexane in a Soxhlet apparatus to eliminate chlorophylls and fatty components. The remaining vegetal material was then extracted successively with solvents of increasing polarity; i.e. first benzene and then ethyl acetate. Each extraction was performed using 180 mL of each solvent during 8 h. This length of time ensures the exhaustion of the vegetable material. The amount of solvent and sample used in the extractions was determined by the dimensions of the Soxhlet apparatus [25]. The extracts obtained were dried in vacuo. The concentration of each AQ in the extracts was determined by High-performance liquid chromatography (HPLC).

2.4. Ultrasonic-assisted extraction

The ultrasonic irradiation experiments were carried out in a TESTLAB SRL sonomatic cleaning bath (model- TB02TACF) operating at 80 W power and 40 kHz frequency. The dimensions of the tank were 150 × 140 × 100 mm. The same solvents used for the conventional extraction were employed. Stems (1 g) and leaves (1 g) of *H. pustulata*, mechanically triturated, were extracted separately in this apparatus. First the plant material was subjected to one hour extraction with 20 mL of hexane to eliminate chlorophylls and fatty components. The remaining vegetable material of each sample (stems and leaves) was extracted at a constant temperature of 50 °C (maximum temperature of the ultrasonic bath) during 15, 30, 45 and 60 min, using 20 mL of benzene. Each sample was then extracted with ethyl acetate, after being treated with benzene for 1 h. The parameters (temperature, amount of solvent, extraction time and number of replications) applied in the ethyl acetate extractions were the same as those for benzene. These experiments were performed by triplicate. In this case the ratio solvent/sample was 20:1, in accordance with that previous literature [26]. Finally, the extracts were filtered and dried in vacuo. The concentration of each AQs in the extracts was determined by HPLC.

2.5. Microwave-assisted extraction

MAE experiments were performed in a microwave oven BGH Litton 16650 (900 W maximum power), equipped with a hermetic glass reactor (Schott, 50 mL capacity) having a Teflon close and a temperature sensor. It is widely accepted that non-polar solvents, like hexane and benzene, will remain transparent to microwaves; thus producing no heat [27,28]. Therefore, previously to MAE extraction with ethyl acetate, 0.3 g of each part of plant (stems and leaves) was pretreated during 1 h with UAE, using hexane and benzene successively, under the same conditions mentioned in Section 2.4. Afterwards, 0.3 g of each remaining material (stems and leaves) was treated with 6 mL of ethyl acetate in the microwave. Again, a 20:1 solvent to sample ratio was applied. In order to evaluate the optimal conditions for microwave extractions, the samples were treated with different irradiation powers (50, 70, 100%; i.e. 450 W, 630 W and 900 W) and times (15, 30, 60 min). The extracts were then filtered and dried in vacuo. The concentration of each AQs in the extracts was determined by HPLC.

2.6. High-performance liquid chromatography

The dried extracts obtained in each experiment were dissolved in methanol (MeOH, HPLC grade). All samples were filtered through a 0.2 mm cellulose acetate membrane filter (Micro Filtration System) before HPLC analysis. HPLC analysis (qualitative and quantitative) was performed in a Varian Pro Star chromatograph (model 210, series 04171), equipped with a UV–Vis detector and a Microsorb-MV column 100–5 C_{18} (250 × 4.6 mm i.d., Varian). The mobile phase was MeOH–H_2O (8:2) at constant flow (1 mL/min) and the injection volume was 20 µl. The detection was performed at the wavelength of 269 nm. The AQs were identified by comparison of the HPLC retention times (t_R) with the corresponding standards (rubiadin, soranjidiol y rubiadin 1-methyl ether) under the same chromatographic conditions.

The external calibration method was carried out to quantify each AQ in every extract, by interpolating the area under each peak (AUP) for each compound from the calibration curves [29]. The seven-points calibration curves ($n = 3$) were linear (correlation coefficients > 0.99).

3. Results and discussion

3.1. Ultrasonic-assisted extraction

3.1.1. Effect of time on the amount of extract

The effect of time on the amount of total extract obtained was analyzed. Figs. 1a and 1b reports the percentage of extract obtained from leaves and stems of *H. pustulata* with each solvent (benzene and ethyl acetate) as a function of time. The data are given as a mean value ± the standard deviation (SD).

For both materials plant (stems and leaves) and solvents (benzene and ethyl acetate), the amount of extract increases with time, showing a sharp increase from 0 to 15 min and then a second increase from 45 to 60 min. This can be due to a two-stage mechanism of extraction. The first stage corresponds to a washing period, in which a fast transfer of the solute takes place from the solid surface and from the outer broken cells to the solvent. The second stage is characterized by a slower diffusion process in which the solute is transferred from the inside of the solid to the solvent. [30].

For both solvents, the amount of extract obtained from the leaves was greater than that from the stems. This phenomenon could be explained by a greater intensification of the mass transfer in leaves than in stems due to a greater enlargement of the pores of the cell walls in the leaves, produced by cavitational effects [31,32].

3.1.2. Chemical composition of the extracts

Identification of AQs in each extract was done by HPLC. Fig. 2 exhibits a typical chromatogram obtained in the UAE experiments. The particular chromatogram shown in this figure corresponds to an extract obtained by UAE during 30 min using benzene as solvent and stems as plant material. In this chromatogram, the peaks identified correspond to rubiadin ($t_R = 6.6$ min), soranjidiol ($t_R = 10.6$ - min) and rubiadin 1-methyl ether ($t_R = 5.5$ min).

Quantification of each of these three AQs was completed using the linear calibration curves. As shown in Table 1, in all cases the total percentage of AQs increased with extraction time, reaching a maximum at 45 min. After this, the concentration of AQs in the extract decreases, despite the remarkable growth in the total amount of extract obtained from 45 to 60 min (as discussed in Section 3.1.1). Thus, this result shows that the increment in the amount of extract is mainly due to the solubilization of non-anthraquinone compounds such as flavonoids and iridoids [8], which show a lower diffusion rates. In addition, it can be observed that, for both solvents tested, the percentage of total AQs is greater in stems than in leaves for all times analyzed ($p < 0.01$).

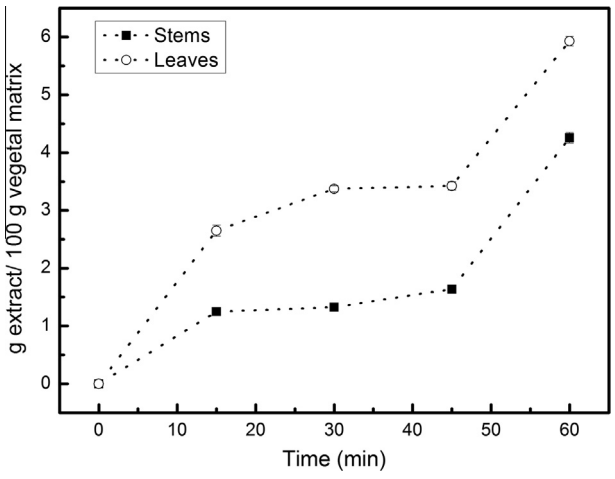

Fig. 1a. Extract obtained as a function of time in benzene.

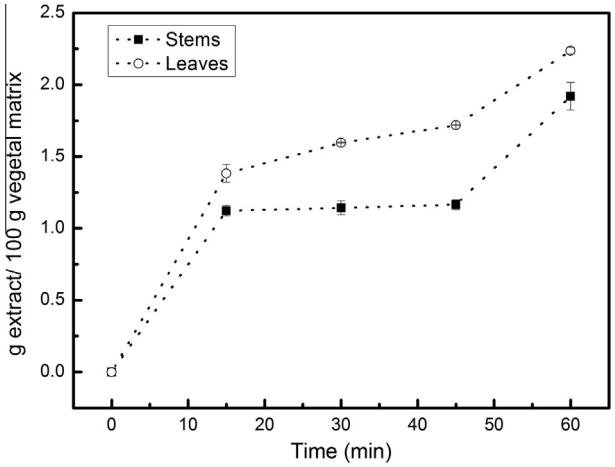

Fig. 1b. Extract obtained as a function of time in ethyl acetate.

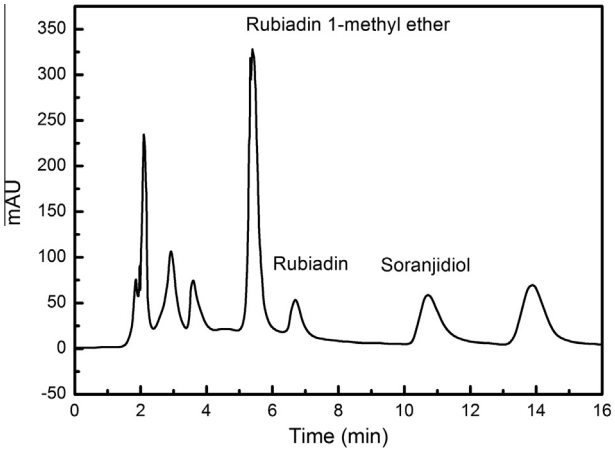

Fig. 2. Qualitative HPLC analysis of extract of stems obtained by UAE using benzene at 30 min.

3.1.3. Selection of the optimal UAE conditions

To define the optimal UAE extraction time, which is important when performing successive extractions with solvents of increasing polarity, it is necessary to determine and compare the yield and selectivity achieved in each extraction.

The yield is determined from the ratio between the amount of the desired compound (AQs) and the initial amount of plant material (stems or leaves). The selectivity is defined as the ratio between the amount of the desired compound (AQs) and the total amount of extract [33].

Fig. 3a shows, for both plant materials (stems and leaves) and for each solvent, benzene (Fig. 3a) and ethyl acetate (Fig. 3b), the yield (% AQ/vegetal matrix) and selectivity (% AQ/extract) of totals AQs (sum of the three analyzed AQs) as a function of time. The figure shows that the higher yield is obtained at 1 h. However, the optimal selectivity is reached at 45 min, indicating that at this time an extract more pure in AQs is obtained.

Table 1
Amount of AQs in the extracts (g/g) obtained in the UAE experiments with stemes and leaves of *H. Pustulata*.

Time(min)	Rubiadin (g/g of extract)	Soranjidiol (g/g of extract)	Rubiadin 1-methyl ether (g/g of extract)	Total(g AQs/g of extract)
Stems*				
Benzene				
15	0.0314 ± 0.0075	0.0135 ± 0.0180	0.0335 ± 0.0310	0.0785 ± 0.0018
30	0.0293 ± 0.0087	0.0119 ± 0.0183	0.0277 ± 0.0350	0.0689 ± 0.0206
45	0.0892 ± 0.0142	0.0284 ± 0.0119	0.0743 ± 0.0661	0.1921 ± 0.0307
60	0.0774 ± 0.0077	0.0169 ± 0.0141	0.0417 ± 0.0190	0.1361 ± 0.0136
Ethyl acetate				
15	0.1321 ± 0.0184	0.0256 ± 0.0193	0.0667 ± 0.0565	0.2244 ± 0.0314
30	0.1356 ± 0.0135	0.0193 ± 0.0261	0.0614 ± 0.0253	0.2163 ± 0.0216
45	0.1981 ± 0.0237	0.0167 ± 0.0361	0.0573 ± 0.0382	0.2722 ± 0.0326
60	0.0904 ± 0.0072	0.0115 ± 0.0125	0.0320 ± 0.0124	0.1340 ± 0.0107
Leaves				
Benzene				
15	0.0032 ± 0.0121	0.0160 ± 0.0158	0.0008 ± 0.0070	0.0201 ± 0.0116
30	0.0044 ± 0.0083	0.0146 ± 0.0045	0.0006 ± 0.0143	0.0196 ± 0.0090
45	0.0057 ± 0.0134	0.0167 ± 0.0095	0.0008 ± 0.0189	0.0232 ± 0.0139
60	0.0059 ± 0.0098	0.0133 ± 0.0036	0.0004 ± 0.0126	0.0197 ± 0.0086
Ethyl acetate				
15	0.0085 ± 0.0175	0.0321 ± 0.0081	0.0017 ± 0.0253	0.0424 ± 0.0169
30	0.0061 ± 0.0196	0.0355 ± 0.0090	0.0010 ± 0.0353	0.0426 ± 0.0213
45	0.0085 ± 0.0231	0.0365 ± 0.0123	0.0009 ± 0.0531	0.0461 ± 0.0295
60	0.0068 ± 0.0076	0.0011 ± 0.0018	0.0011 ± 0.0030	0.0091 ± 0.0041

* $p < 0.01$ Measures with respect to leaves (%AQs/leaves).

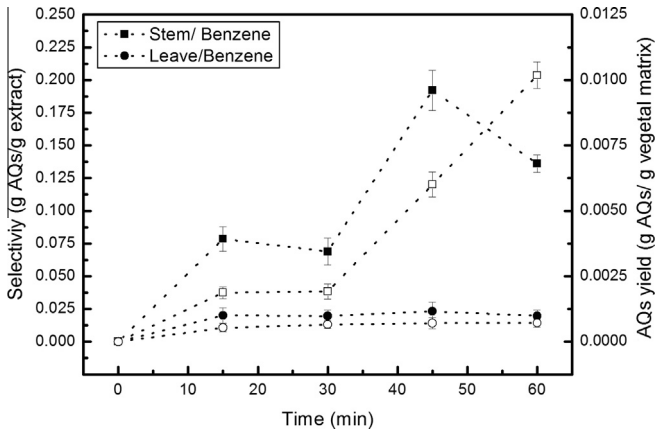

Fig. 3a. Selectivity (g AQs/g extract; solid symbols) and AQs yield extraction extracted (gAQs/100 g vegetal matrix; open symbols) of stems and leaves in benzene.

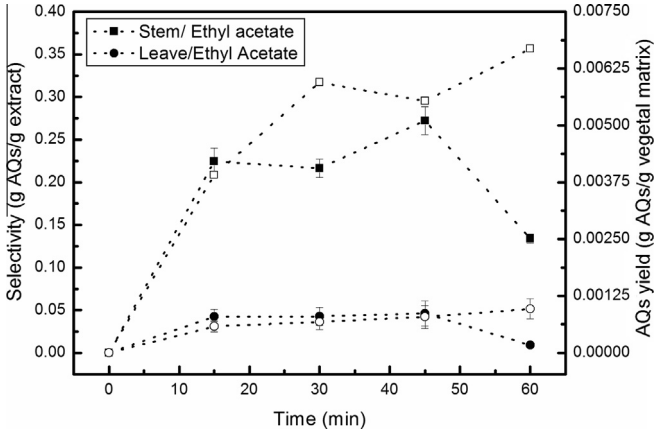

Fig. 3b. Selectivity (g AQs/g extract; solid symbols) and AQs yield extraction extracted (gAQs/g vegetal matrix; open symbols) of stems and leaves in ethyl acetate.

In addition, Fig. 3b displays that at 1 h the yield for stems has an increasing trend, while that for leaves has a constant tendency, indicating that in this case the plant material is exhausted. In conclusion, we consider that the optimum extraction time would be 1 h, since a balance is reached between yield and selectivity. Whereas an increase in extraction time would give a higher yield (especially for stems), there will be a marked decrease in selectivity; hence, requiring a more difficult purification step.

3.2. Microwave-assisted extraction

AQs extraction with ethyl acetate by MAE was performed at a constant power of 450 W, 630 W and 900 W (50, 70 and 100%). Under these conditions, the temperature of the solvent increased with time as shown in Fig. 4.

The maximum AQs yield was achieved at 15 min at the higher power (100%), at 30 min for the 70% power and at 60 min for the lower power (50%) (see Fig. 5 and Table 2). This is an expected result, as the higher power will produce a faster breakdown of the cell walls, providing an easier access of the solvent to the AQs [34,35]. Therefore, optimum conditions to obtain the highest yield of AQs were 100% microwave power during 15 min. From Fig. 4 it can be seen that, for the three powers studied, the temperature at the conditions of maximum yield was around 69.5 ± 0.3 °C. Regarding selectivity, the concentration of AQs in the extract followed the same behavior as the yield, reaching a maximum value at 15 min, 30 min and 60 min for the 100%, 70% and 50% power, respectively (see Table 2).

3.3. Comparison of extraction methods

Soxhlet, UAE and (UAE + MAE) extractions were investigated to determine the best technique for extracting AQs from stem and leaves of *H. pustulata*. Table 3 summarizes the results obtained with the three methods, including the yield of total AQs obtained (rubiadin + soranjidiol + rubiadin 1-methyl ether) for both parts

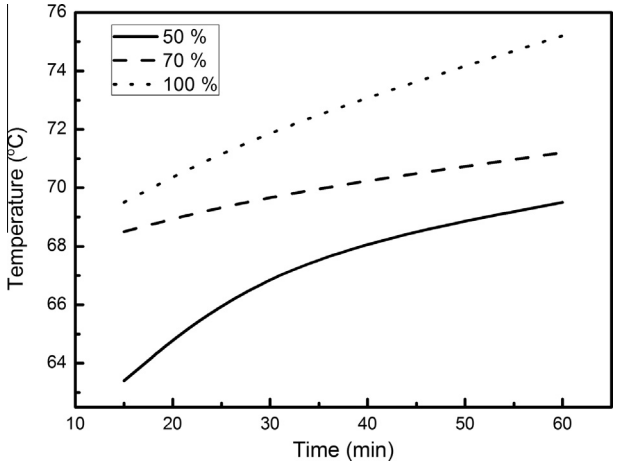

Fig. 4. Ethyl acetate temperature as a function of time at different microwave power.

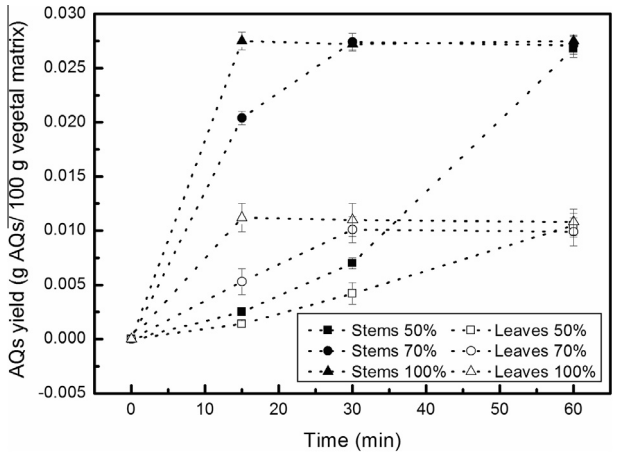

Fig. 5. AQs yield extraction (gAQs/g vegetal matrix) as a function of time for different powers in MAE.

of the plant (stems and leaves) by adding both extraction solvents (benzene plus ethyl acetate). In addition the same table reports the time of extraction, amounts solvent used by unit of sample, efficiency (yield of total AQs obtained from the plant/time of extraction) and energy consumption by unit of mass of obtained AQs.

All these data were determined at the optimal time for each solvent used: 8 h benzene + 8 h ethyl acetate for Soxhlet, 1 h benzene + 1 h ethyl acetate for UAE and 1 h benzene + 15 min ethyl acetate at 100% power for UAE plus MAE. The analysis of this table show that the Soxhlet conventional extraction process has a low extraction yield of total AQs: 0.0034 g/g vegetal matrix for stems and 0.0012 g/g vegetal matrix for leaves, requiring long times and large amounts of solvent.

The UAE increased the extraction yield in stems and leaves: 0.0168 g/g vegetal matrix and 0.0016 g/g vegetal matrix respectively, reducing time and amount of solvent. However, the method that combines a UAE pretreatment with benzene, followed by MAE with ethyl acetate at a constant power, gave the best results, producing the higher yield of total AQs in stems and leaves (0.0239 g/g vegetal matrix and 0.0063 g/g vegetal matrix, respectively), in less time and using the same amount of solvent that UAE.

The total energy consumption by unit of mass of obtained AQs of each technique reported in Table 3 was calculated taking into account the power and time at which maximum performance was achieved. Soxhlet energy consumption was remarkably higher than those obtained by MAE and UAE, which exhibited similar values.

UAE and UAE + MAE showed a high extraction yield of AQs compared to Soxhlet (see Fig. 6 and Table 3). It can also be clearly observed that the yields obtained from stems are superior with regard to those obtained from leaves. In the same figure it can be seen that the new techniques proposed favored the extraction yield of AQ with ethyl acetate, in all cases.

Finally, Fig. 7 shows the percentage of each ratio AQs/vegetal matrix (stems and leaves), calculated as the amount of each AQ extracted by both solvents under the optimal conditions selected for each technique. This figure shows that, for stems, the extraction of the three AQs, mainly soranjidiol and rubiadin 1-methyl ether, was improved by the new techniques. However, for leaves the amount of these compounds extracted was only higher by using UAE + -MAE. Comparing the two alternative techniques, we can conclude that UAE in combination with MAE is the method of choice, since the extracted amount of the three compounds is greater than for UAE for both plant parts, highlighting again soranjidiol and rubiadin 1-methyl ether as the main AQs extracted.

4. Conclusions

UAE and UAE + MAE have proved to be an attractive alternative to the conventional Soxhlet extraction methodology for AQs. The combination of UAE + MAE showed to be the best alternative, since it exhibited the highest efficiency (see Table 3) and promoted a larger extraction of rubiadin, soranjidiol and rubiadin 1-methyl ether; the latter two in greater proportion. The optimal conditions to obtain the maximum yield of AQs with this technique were: UAE using benzene at 50 °C during 60 min, followed by MAE using ethyl acetate as extraction solvent at 900 W constant power for 15 min. These results demonstrate that the combination UAE + MAE is a

Table 2
Amounts of AQs in extract and vegetal matrix extracted with ethyl acetate in MAE at constant power.

Power (%)	Time (min)	Stems*		Leaves	
		(g AQs/g of extract)	(g AQs/g of vegetal)	(g AQs/g of extract)	(g AQs/g of vegetal)
50	15	0.0965 ± 0.0212	0.0010 ± 0.0002	0.0090 ± 0.0042	0.0005 ± 0.0003
50	30	0.1565 ± 0.0250	0.0035 ± 0.0005	0.0240 ± 0.0116	0.0020 ± 0.0010
50	60	0.4700 ± 0.0282	0.0135 ± 0.0008	0.0500 ± 0.0110	0.0050 ± 0.0011
70	15	0.3960 ± 0.0237	0.0100 ± 0.0006	0.0340 ± 0.0164	0.0025 ± 0.0012
70	30	0.4770 ± 0.0286	0.0135 ± 0.0008	0.0530 ± 0.0127	0.0050 ± 0.0012
70	60	0.4720 ± 0.0283	0.0135 ± 0.0008	0.0515 ± 0.0143	0.0045 ± 0.0013
100	15	0.4820 ± 0.0289	0.0135 ± 0.0008	0.0545 ± 0.0131	0.0055 ± 0.0013
100	30	0.4755 ± 0.0190	0.0135 ± 0.0005	0.0520 ± 0.0156	0.0050 ± 0.0015
100	60	0.4790 ± 0.0191	0.0135 ± 0.0005	0.0525 ± 0.0136	0.0045 ± 0.0012

* $p < 0.01$ Measures with respect to leaves.

Table 3
Comparison of extraction methods analyzed.

Extraction methods	Vegetal matrix	Yield total AQs (g/g of vegetal matrix)	Time (h)	Solvent consumption by unit of sample (ml/g)	Efficiency (g/h)	Energy consumption (kJ/gAQs)
Soxhlet	Stems	0.0034[a]	16	36	0.00021	6260869
	Leaves	0.0012[a]	16	60	0.00007	28800000
UAE	Stems	0.0168[b]	2	20	0.00840	857143
	Leaves	0.0016[b]	2	20	0.00165	8727272
UAE + MAE	Stems	0.0239[c]	1.25	20	0.01912	3828452
	Leaves	0.0063[c]	1.25	20	0.00504	14523809

[a] Time: 8 h benzene + 8 h ethyl acetate.
[b] Time: 1 h benzene + 1 h ethyl acetate.
[c] Time: 1 h benzene + 15 min ethyl acetate at 100% power.

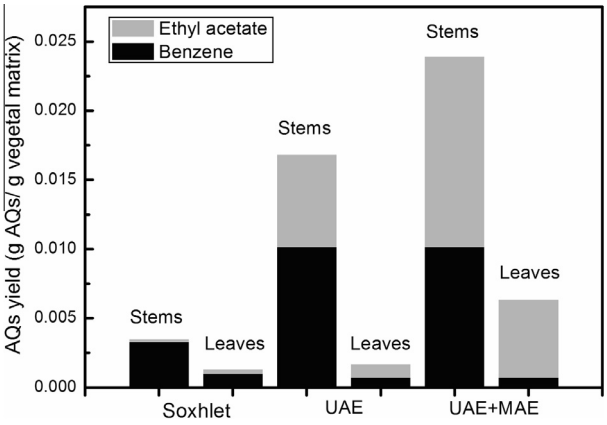

Fig. 6. Comparison of total AQs yield extraction using different extraction methods for leaves and stems, in benzene and ethyl acetate.

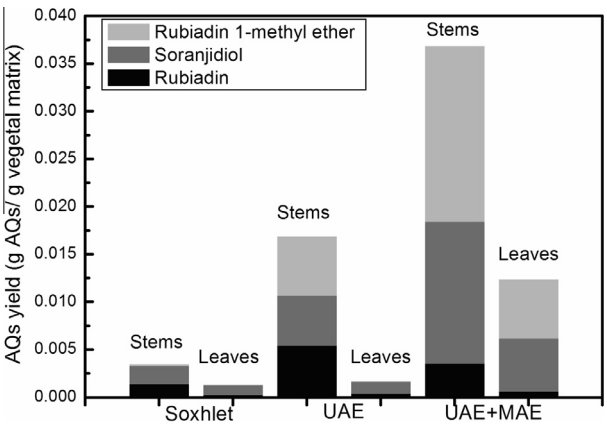

Fig. 7. AQs yield extraction: AQs extracted from leaves and stems summing both solvent at optimal conditions for each technique used.

potentially useful technique to obtain better AQs-rich extracts from *H. pustulata*.

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