Industrial Crops and Products 69 (2015) 278–283

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Ultrasonic-assisted extraction of anthraquinones from *Heterophyllaea pustulata* Hook f. (Rubiaceae) using ethanol–water mixtures

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ARTICLE INFO

Article history:
Received 8 September 2014
Received in revised form 9 December 2014
Accepted 30 January 2015

Keywords:
Ultrasound-Assisted Extraction
Anthraquinone
Optimization
Taguchi experimental design

ABSTRACT

In this study, ultrasound-assisted extraction (UAE) was evaluated as an easier and more efficient alternative method for the isolation of anthraquinones (AQs) from *Heterophyllaea pustulata* Hook f. (Rubiaceae), compared with conventional extraction techniques. The extraction was performed using an ethanol–water solution as solvent. The influence of different operating conditions, as temperature, time, solvent composition, and solvent/sample ratio and its relationship with ultrasonic techniques was analyzed. For this purpose and to achieve global optimization of the ultrasound-assisted extraction of *H. pustulata*, Taguchi experimental design with a L9 orthogonal array was applied.

The results showed that the yield obtained with the ultrasound assisted extraction is approximately twice higher than the traditional Soxhlet technique, and Taguchi analysis demonstrated that the most influential factor is solvent concentration, followed by time, temperature, and solvent/sample ratio. The solvent composition effect is directly related to cavitation process, and the effect of solvent physical properties on it. The optimal combination of these factors on the ultrasound assisted extraction is a solvent composition 60% v/v of ethanol, a solvent/sample ratio equal to 20:1, a temperature of 55 °C, and an extraction time of 30 min.

1. Introduction

Anthraquinones (AQs) are an important group of secondary metabolites and constitute the largest group of natural quinones. AQ derivatives are chemotaxonomic markers in certain plants families: Rubiaceae, Rhamnaceae, Polygonaceae, Fabaceae, Liliaceae, and Verbenaceae (Wijnsma et al., 1986). To this group belongs the Rubiaceae species *Heterophyllaea pustulata* Hook. f., commonly known as "cegadera", "ciegadera" or "saruera". This plant grows spontaneously in the mountainous regions of NW Argentina and Bolivia, between 2500 and 3000 m over sea level (Bacigalupo and Cabrera, 1993). The first chemical investigations of *H. pustulata* were performed by our researcher group (Nuñez Montoya et al., 2003; Núñez Montoya et al., 2008), where the presence of several chemicals components (anthraquinones, flavonoid, and iridoid) was established, with a significant predominance of aglycone- 9,10-anthraquinones (AQs). Thus, from the aerial parts of *H. pustulata* three flavonoids (quercetin, isoquercitrin, quercetin-3-*O*-α-D-glucosyl-6"-acetate), an glicosyde iridoid (asperuloside), ten aglycone-AQs: soranjidiol, soranjidiol 1-methyl ether, rubiadin, rubiadin 1-methyl ether, damnacanthal, damnacanthol, heterophylline, pustuline, 5,5-bisoranjidiol, and 2-hydroxi-3-methyl anthraquinone were isolated. The first nine AQs have 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) (Núñez Montoya et al., 2005; Comini et al., 2007). Three of these AQs, soranjidiol, rubiadin and 1-methyl ether rubiadin, stand out as the main components of leaves and stems (Nuñez Montoya et al., 2003). These AQs have demonstrated important antibacterial and anticancer activity *in vitro* by means of the photosensitizing phenomenon (Comini et al., 2011a,b). In addition, we have previously established that extracts containing these compounds exhibited a significant antibacterial, antifungal and antiviral activity *in vitro*

Abbreviations: AQs, anthraquinones; UAE, Ultrasound assisted extraction; O_2^-, superoxide anion; ^{1}, O_2singlet molecular oxygen; HPLC, High-performance liquid chromatography.

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http://dx.doi.org/10.1016/j.indcrop.2015.01.065
0926-6690/

without the involvement of an photosensitizing action (Konigheim et al., 2012; Nuñez Montoya et al., 2003). Due to the interesting biological activitys presented by these AQs and their wide range of applications in the pharmaceutical industry, is of great interest to optimize the processes to extract these compounds.

Traditional extraction methods for AQs, involve the use of a Soxhlet apparatus with organic solvents of increasing polarity, starting with hexane, followed by benzene, ethyl acetate, and ethanol (Nuñez Montoya et al., 2003). Although this multiple-solvent extraction system is faster, less laborious, and consumes less amount of solvent than other conventional methods (maceration, reflux, etc.), it presents low selectivity for certain secondary metabolites, such as AQs from *H. pustulata*. Furthermore, benzene and hexane are hazardous substances, due to its toxicity and flammability. Therefore, it is important to develop more selective and efficient techniques for obtaining AQs, using less harmful solvents and requiring lower extraction times. In this sense, ultrasound-assisted extraction (UAE) is a very interesting alternative for the extraction and purification of these substances. It has been widely used to isolate bioactive substances from plants (Rostagno et al., 2003; Vilkhu et al., 2008; Vinatoru, 2001). All published studies of ultrasound extractions indicate higher yields as well as shorter extraction times (Bagherian et al., 2011; Vilkhu et al., 2008). The larger extraction efficiency is mainly due to cavitation effects, which improve mass transfer and solvent penetration in the plant material by disrupting the cell walls (Knorr, 2003).

Hemwimol et al. (Hemwimol et al., 2006), have applied UAE to extract AQs from the roots of *Morinda citrifolia.* These authors studied the effects of different extraction conditions: temperature, ultrasonic power, and solvent types. The results show that the yield increases with increasing extraction times, extraction temperatures, and the use of ethanol–water solutions as extraction solvent.

In a previous work (Hemwimol et al., 2006), UAE was also applied for the extraction of AQs from the stems and leaves of *H. pustulata*, in which this method was compared with MAE and Soxhlet, using a sequence of solvents with increasing polarity (hexane, benzene, and ethyl acetate). The results showed that UAE increases the extraction yield of total AQs and reduces the time and amount of solvent used. Nevertheless, the combination of UAE with benzene, plus MAE with ethyl acetate at a constant power, gave the best results.

In this work UAE of AQs from *H. pustulata* using ethanol–water mixtures as solvent is studied, in order to replace harmful solvent used in traditional extractions. Ethanol is a non-toxic and economic solvent widely used in the extraction of active principles from plants (Barrera Vázquez et al., 2014). Being a polar organic solvent, ethanol is in principle an adequate solvent for the slightly polar AQs molecules.

In order to investigate the influences of different factors on extraction, including solvent composition, solvent/sample, temperature, and extraction time a mathematical modeling was applied. Taguchi method has been widely used in many applications of engineering, as it is a powerful tool to design and investigate the influence of different variables. It has a special design of an orthogonal matrix to study factors, in order to minimize the number of tests and, consequently, the time and costs experimental (Taguchi and Konishi, 1987). While different mathematical models have been applied to UAE optimize of natural compounds (Sahin and Saml, 2013; Tabaraki and Nateghi, 2011; Wang et al., 2013), many studies have applied the Taguchi design (Chuichulcherm et al., 2013; Rouhani et al., 2009). Yet there are no reports on the optimization of UAE for extraction of AQs.

Four factors (ethanol–water solvent composition, solvent/sample ratio, temperature, and time) were evaluated to determine the optimal extraction conditions, by using L9 Taguchi orthogonal design. This study was complemented with an ANOVA variance analysis, to determine the statistically significant factor. The results obtained by UAE were compared with those obtained with the conventional Soxhlet extraction method.

2. Materials and Methods

2.1. Plant material

Aerial parts of *Heterophyllaea pustulata* were collected in La Almona, Jujuy province, Argentina, in January 2011. The vegetal specie 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. The plant material was air-dried and then was separated into stems and leaves.

2.2. Solvents

The solvents used in the extractions were: ethanol (Porta, 96% v/v) and distilled water.

2.3. Ultrasonic-assisted extraction.

Stems of *H. pustulata* (0.25 g) were air-dried. Then, these stems were triturated mechanically using a knife mill (Retsch K.G. 5657 Haan West-Germany) with a mesh n° 5 (sieve opening 4 mm). After this pretreatment, these stems were submitted to Ultrasonic-assisted extraction by using ethanol–water solutions.

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. Dimensions of the tank were 150 × 140 × 100 mm.

The extraction of the plant material was evaluated at different conditions: solvent composition (60, 80, and 96% v/v of ethanol), temperatures (35, 45, and 55 °C), extraction time (15, 30, 45 min), and solvent/sample ratio (10:1, 20:1, and 30:1). These experiments were performed by triplicate. Finally, the extracts were filtered and dried under vacuum. The concentration of AQs in the extracts was determined by High-Performance Liquid Chromatography (HPLC).

2.4. Conventional Soxhlet extraction

Dried stems (29 g) were mechanically triturated and treated with an ethanol–water solution (435 ml), in a concentration that was selected from the results obtained in the UAE experiments, which correspond to the ratio with greater extractive capacity (60% v/v of ethanol). The amount of solvent and sample used in these extractions was determined by the dimensions of the Soxhlet apparatus (Cela et al., 2002), whereas the period of time used (9 h) ensured the exhaustion of the vegetable material (Núñez Montoya et al., 2008). The extracts obtained were dried under vacuum. The concentration of AQs in the extracts was determined by HPLC.

2.5. 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. Qualitative and quantitative analysis was performed in a Varian Pro Star chromatograph (model 210, series 4,171), 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

Table 1
Factors and levels for the orthogonal design (A–D are the respective codes for each factor).

Levels	Factors			
	[A] Solvent composition (% v/v of ethanol)	[B] Ratio solvent/sample	[C] Temperature (°C)	[D] Time (min)
1	96	10	35	15
2	80	20	45	30
3	60	30	55	45

of 269 nm. Rubiadin, soranjidiol, rubiadin 1-methyl ether, and 2-hydroxi-3-methyl anthraquinone were identified by comparison of the HPLC retention times with authentic samples previously obtained by our laboratory (Nuñez Montoya et al., 2003), under the same chromatographic conditions.

The external calibration method was applied to quantify each AQ in every extract, by interpolating the area under each peak for each compound from the calibration curves (Nuñez Montoya et al., 2008). Seven-points calibration curves ($n = 3$) were linear (correlation coefficients >0.99).

2.6. Experimental Design

The experiments were designed to examine the effect of extraction factors and to optimize the operating conditions for the extraction of AQs with ethanol–water solutions. In order to optimize the extraction conditions, the Taguchi-based optimization technique was adopted.

In this study, four control factors (solvent composition [A], solvent/sample ratio [B], temperature [C], and time of ultrasonic irradiation [D]), with three levels setting, were considered to be the independent variables and they are summarized in Table 1. For the process optimization a L9 orthogonal matrix was adopted. The rows of the matrix represent 9 experimental runs which were carried out in a random sequence (Taguchi and Konishi, 1987). The results of extractions performed under orthogonal design conditions are shown in Table 2.

Each experiment was repeated three times under the same conditions at different times, to observe the effects of noise sources in the extraction yield. All the results at each step of the design are expressed as the mean value of three experiments. Mean value of these replications is the response of this treatment.

After conducting the experiments, the results were converted into a statistical measure of performance, represented by the signal-to-noise (S/N) function. There are three S/N ratios of common interest for optimization of statistic problems: the lower-the-better, the higher-the-better and the nominal-the-best (Ranjit, 2001). In our case the experimental objective was the yield of AQs, so the higher-the-better option was applied:

$$\frac{S}{N} = -10 \times \log(M.S.D.) \quad (1)$$

Table 2
The results of orthogonal test L9 (3^4).

Test	A	B	C	D	(mg AQs/g of vegetal)	S/N ratio
1	1	1	1	1	1.13	0.91
2	1	2	2	2	1.56	3.58
3	1	3	3	3	1.66	4.19
4	2	1	2	3	2.97	8.09
5	2	2	3	1	2.74	8.02
6	2	3	1	2	2.79	8.89
7	3	1	3	2	3.01	9.37
8	3	2	1	3	2.98	8.97
9	3	3	2	1	2.43	7.27

$$M.S.D = \frac{1}{m}\sum_{i=1}^{m}\frac{1}{T_i^2} \quad (2)$$

where m is the number of tests and T_i is the value of the yield of AQs in the i test.

The importance of each factor is studied in the signal to noise (S/N) ratio by considering both, the mean (signal) and the standard deviation (noise) (Ranjit, 2001; Yang and Tarng, 1998).

2.7. Statistical analysis

The purpose of the analysis of variance (ANOVA) is to investigate the design factors that significantly affect to extraction of AQs. Statistical analyses (ANOVA) were performed using Minitab 15. The F-value standard for a statistical confidence level of 90%. All analyses were performed in triplicate.

3. Results and discussion

3.1. Optimization of extraction conditions. S/N ratio analysis

The effect of different process variables was analyzed by Taguchiís method as explained above.

The experimental results shown in Table 2 are transformed into a signal-to-noise (S/N) function to analyze the effects of each factor and level.

The power and frequency are important operating conditions in the UAE, however, according of the available equipment, in this study, these conditions (power and frequency) were maintained constant during the test.

To analyze the data using the S/N function, Taguchi suggests making a table of responses, to identify and isolate the effects of each factor on S/N. The average of S/N for each level and factor are shown in Table 3. The relative difference indicates that the solvent composition factor (A) has an important influence on the yield of AQs, followed by time (D), temperature (C) and solvent/sample ratio (B) in decreasing order. The optimal level for each factor corresponds to the average maximum; therefore, the optimal condition for the extraction of AQs is A_3 B_2 C_3 D_2 (ethanol/water composition = 60:40 (v:v), solvent/material ratio = 20:1, temperature = 55 °C and time = 30 min). The contributions of the different levels of each factor in the yield of AQs, can also be visualized in Fig. 1.

This optimal configuration is not found in the L9 array (see Table 2). This is one of the properties of Taguchiís method, which can detect untested configurations. The predicted S/N ratio and AQs yield using the optimal level of the design factors can be calculated as:

$$[Y]_{\text{predicated}} = \mathrm{T}m + \sum_{i=1}^{m}([Yji]_i - [\mathrm{T}m]) \quad (j = \mathrm{A}, \mathrm{B}, \mathrm{C}, \mathrm{D}) \quad (3)$$

where Tm is the total mean of S/N ratio or total mean of AQs yield and $[Yji]_i$ is the S/N ratio or AQs yield at the optimal level (Ranjit, 2001).

Table 3
S/N Response Table.

	A	B	C	D
Levels 1	2.89	6.13	6.26	5.4
Levels 2	8.34	6.86	5.31	7.28
Levels 3	8.54	6.78	7.2	7.09
Delta	5.64	0.73	0.93	1.88
Effect	1	4	3	2

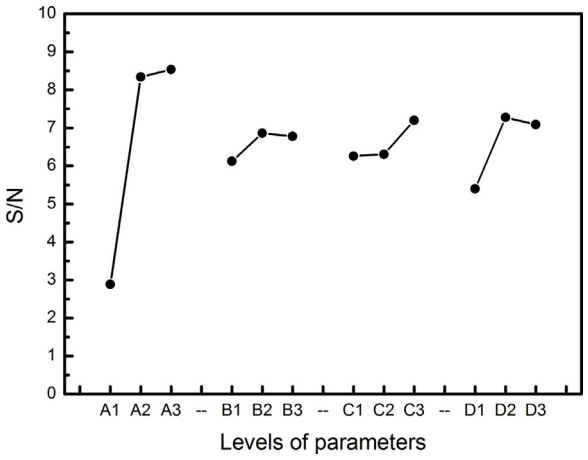

Fig. 1. Effect of different levels of L9 orthogonal array (Taguchiís experimental design) for each parameter on the AQs yield.

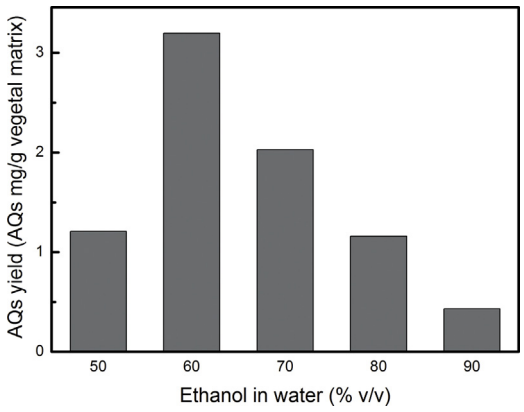

Fig. 2. Effect of solvent composition on the UAE. Extraction conditions: time 30 min; solvent/sample ratio 20:1; temperature 55 °C.

Using Eq. (3), the predicted value of S/N is 10.11 and the predicted yield is 3.07 mg AQs/g plant material.

The purpose of these calculations is to validate the experiment. The validation experiments were repeated 3 times under the optimized conditions, obtaining an S/N ratio of 10.90 and a AQs yield of 3.28 mg AQs/g plant material. This indicates that there is a good agreement between the predicted and the experimental recovery at the optimal process conditions (A_3 B_2 C_3 D_2).

Statistical analysis of variance (ANOVA) was performed to see whether the process parameters are statistically significant. The *F*-value for each parameter indicates which parameter has a significant effect on the extraction and is simply the ratio between the variance due to the effect of a factor and the variance due to the error term. The *F*-value obtained for each factor is compared with the value of *F*-standard tables (Fα) for a given statistical level of significance.

The *F*-value of each factors was calculated and compared to the critical Fα value read from the distribution table of *F*-values standard (Ross, 1988) for inspection level $\alpha = 0.10$ ($F0.1(2,18) = 2.64$). The factor effect is prominent if *F*-value is larger than Fα. As seen in Table 4, it is evident that factor A is statistically significant for the extraction of AQs (FA > Fα,), while factors B, C and D have no significant effect (FB, FC, and FD < Fα) at the conditions explored. Furthermore, from the rate of contribution P% (Table 4), it may also be deduced that the most important factor which contributes to the extraction yield of AQs is solvent concentration (22.56%), followed by extraction time (1.95%) and temperature (0.32%) and finally ratio solvent/sample (0.15%).

3.2. Effect of solvent composition on the extraction of anthraquinones by ultrasound-assisted extraction

After determining the optimal conditions for the extraction of AQs, the effect of solvent composition on the AQ yield was analyzed. Samples of 0.5 g of stems of *Heterophyllaea pustulata* were extracted in the ultrasonic bath at the optimal temperature (55 °C), solvent/sample ratio (20:1) and time (15 min) determined by Taguchiís method, and using different ethanol–water compositions (50, 60, 70, 80, and 96% v/v of ethanol). The different ratios of ethanol–water solutions were considered as solvent for studying the solvent composition effect. Ethanol is a non-toxic and economic solvent widely used in the extraction of active principles from plants (Galvan d'Alessandro et al., 2012). Being a polar organic solvent, ethanol is in principle an adequate solvent for the slightly polar AQs molecules.

As it can be seen in Fig. 2, there is an increase in the AQs yield with the water content in the solvent up to 40% v/v (60% v/v of ethanol) and then a decrease for a 50% v/v mixture. These results are in agreement with the optimal solvent composition determined by Taguchiís method.

Cavitation, which is the main phenomena involved in ultrasound assisted extraction, is affected by certain physical properties of the solvent, such as surface tension, viscosity, and vapor pressure (Asher, 1987; Chivate and Pandit, 1995; Hemwimol et al., 2006; Shah et al., 1999). Fig. 3 shows the value of these properties for ethanol + water mixtures (Chodzińska et al., 2012; Gmheling et al., 1998). Surface tension decreases with the increase in ethanol concentration (Vazquez et al., 1995), while viscosity shows a maximum at about 50% v/v of ethanol (Vazquez et al., 1995) and vapor pressure increases steadily with ethanol concentration till the azeotropic composition (97% v/v at 50 °C). In principle, cavitation would benefit by higher surface tension, lower viscosity, and lower vapor pressure of the cavitating medium (Shah et al., 1999). However, for a given sonic frequency, there is an optimum vapor pressure where the impulse pressure and temperature obtained by the collapse of a cavity are maximum (Chivate and Pandit, 1995). When the vapor pressure is low, few, and small vapor bubbles are produced, but when the vapor pressure is increased beyond the optimum value, large cavities are formed, which, instead of collapsing violently, often disintegrate into smaller cavities or simply dissolve into the solution, lowering the cavitation damage(Chivate and Pandit, 1995).

According to the experimental results, the optimum vapor pressure for the conditions studied corresponds to a solvent with a 60% v/v ethanol. Although viscosity is nearly at its maximum value for this concentration, and the surface tension of the water + alcohol solution is substantially lower than that of pure water, a medium value of the vapor pressure value seems to be the dominant factor in the cavitation effects.

Similar results are reported by Hemwimol et al. (Hemwimol et al., 2006) for AQs extraction from *Morinda citrifolia* roots, using ethanol–water mixtures. These authors found an optimal con-

Table 4
ANOVA SS, sum of square deviation; df, degrees of freedom; *V*, variance; Fj, *F*-ratio; *P*(%), percentage of contribution e, experimental error; T, total of source.

Source	SS	df	*V*	*F*	Fα	*P*(%)
A	3.741	2	1.87	2.707	>	22.56
B	0.025	2	0.012	0.018	<	0.151
C	0.053	2	0.026	0.038	<	0.324
D	0.324	2	0.162	0.235	<	1.959
e	12.435	18	0.690			
T	16.580	26				

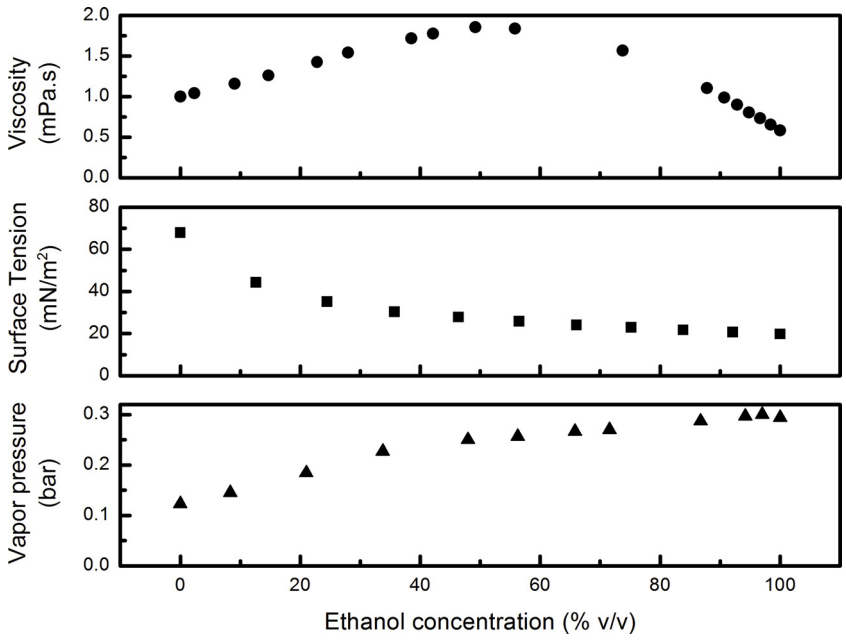

Fig. 3. Effect of ethanol composition on the solvent properties: Viscosity at 293 K (Chodzińska et al., 2012); Surface tension at 323 K (Vazquez et al., 1995); Vapor pressure at 323 K (Gmheling et al., 1998).

centration of 50% of ethanol for ultrasound assisted extraction performed at 25 °C using a power of 15.7 W.

3.3. Effect of time on extraction of anthraquinones by ultrasound-assisted extraction

In the same way, the effect of time was analyzed performing a series of UAE experiments at different times (0, 15, 45, 60, 75, and 90 min), and maintaining the other extraction factors according to the optimal conditions (solvent composition 60% v/v of ethanol, solvent/sample ratio 20:1 and temperature 55 °C). All tests were carried out in triplicate and the results are shown in Fig. 4. As it can be observed, the yield increases with increasing contact time between the plant material and solvent, up to 30 min. After this time, the yield of AQs remains constant (6.56 mg AQs/100 g plant material), indicating that the plant material is exhausted. Again, these results are in accordance with the condition of optimal time determined by Taguchiís method.

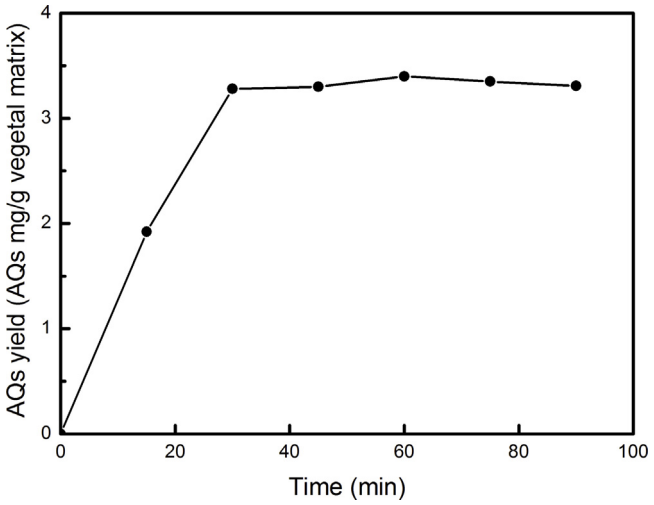

Fig. 4. Effect of time on the UAE. Extraction conditions: solvent composition 60% ethanol; solvent/sample ratio 20:1; temperature 55 °C.

3.4. Comparison of UAE with conventional Soxhlet extractions

In this study, UAE was compared with the conventional Soxhlet extraction technique. The UAE experiments were carried out under the optimal conditions. To compare the two techniques, the Soxhlet extractions were performed using the same solvent composition. The conditions of the different techniques and their results are shown in Table 5.

This Table shows that the total AQs yield obtained by UAE is almost double that obtained by Soxhlet extraction. In addition, it can be noticed that, in both techniques, soranjidiol is the AQ with the major concentration in the extract. With respect to extraction time, UAE is also the fastest extraction method, requiring only 30 min to exhaust the plant material. So, efficiency (yield of total AQs/time of extraction) is greater in UAE than in Soxhlet extraction.

Also, it is important to note that the AQs yield extraction obtained is similar to yield obtained by traditional extraction methods for AQs, wich involve the use of a Soxhlet apparatus with organic solvents of increasing polarity, starting with hexane, followed by benzene, ethyl acetate, and ethanol (Barrera Vázquez et al., 2014). However, the UAE using ethanol presents

Table 5
Comparison of extraction methods analyzed.

Methods	Ethanol (%v/v)	Time(h)	AQs content			Yield total AQs (mg/g og vegetal matrix)	Efficiency (mg AQs/h)
			2-hydroxi-3-methyl anthraquinone	Rubiadin and Soranjidiol	Rubiadin 1-methyl Ether		
UAE	60	0.5	0.77	2.32	1.81	3.28	6.56
Soxhlet	60	9	0.55	0.96	0.74	1.59	0.17

the advantages of lower extraction time (30 min for UAE and 16 h for traditional method) and the use of less harmful solvent.

4. Conclusions

In this work, it was demonstrated that UAE is a useful technique for anthraquinones isolation from *Heterophyllaea pustulata* Hook f. (Rubiaceae). This technique allows obtaining good extraction yield using non harmful solvent and reducing extraction time, due to the improvement of mass transfer by cavitation effects.

The effect of different process variables was analyzed using Taguchiís method with L9 orthogonal array. Based on this study, extraction conditions of AQs were optimized. Thus, it was found that most influential factor in the extraction of AQs is solvent composition, followed by time, temperature and solvent/sample ratio. The solvent composition effect is directly related to the effect of solvent physical properties in cavitation process. This study was completed by the analysis of variance (ANOVA), also indicating that the most statistically significant factor in the extraction of AQs is solvent composition.

The results of the theoretical process optimization gave the following optimal conditions for the AQs extraction: solvent composition 60% v/v of ethanol, solvent/sample ratio 20:1, extraction time 30 min and extraction temperature 55 °C. The experimental yield in the optimal conditions proved to be fairly accurate in predictive yield.

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