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Original Article

Box–Behnken experimental design for extraction of artemisinin from *Artemisia annua* and validation of the assay method

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ABSTRACT

Obtaining artemisinin and its derivatives is very costly, which limits access to low-income people. Some hydroalcoholic extract of *Artemisia annua* L., Asteraceae, which has shown comparable antimalarial activity could be an alternative to the purified compound, especially if the cultivars have higher content of artemisinin. The objective of this study was to evaluate the effects of the extraction parameters (ethanol graduation, previous shaking time in an ultrasound bath and drug/solvent ratio) on the yield of artemisinin in the liquid extract obtained by percolation from *A. annua* and then optimize the extraction efficiency of this compound. The Box–Behnken (3^3) factorial design was used in association with response surface methodology. The derivatization reaction of artemisinin was used in an analytical method which was submitted to validation, after reaching the specification of the selectivity, linearity, precision, accuracy and robustness. Detection and quantification limits were 1.3 and 4.0 μg/ml, respectively. The largest amount of this compound of interest was obtained without any ultrasound bath, with an ethanol graduation of 95% and a drug/solvent ratio of 2%. Drug/solvent ratio was the factor which most influenced extraction efficiency. The maximum range of artemisinin yield was 1.21%. Information obtained in this study can be used for future approaches to determining and extracting artemisinin from *A. annua*.

Introduction

Artemisia annua L., Asteraceae, an annual herbaceous plant, has been used for thousands of years in traditional Chinese medicine to treat fever and malaria without showing any signs of toxicity (Naeem et al., 2014; Van der Kooy, 2014). It has also been widely used as a tea (WHO, 2012) for the treatment and prevention of malaria and to combat other diseases, especially by the people of sub-Saharan Africa (Brisibe and Chukwurah, 2014).

The chemical compound of main therapeutic interest is artemisinin (WHO, 2006), a sesquiterpene lactone containing an endoperoxide ring structure responsible for potent antimalarial activity at nanomolar concentrations (Meshnick et al., 1996). The flavonoid content of the plant drug can vary between 9% and 11% and has already been shown to exert antimalarial and antioxidant activity (Ogwang et al., 2011) and enhance the activity of this lactone (Klayman, 1985; Elford et al., 1987; Bilia et al., 2006).

Obtaining artemisinin and its derivatives is very costly, so this increases the price of the drug on the market and limits access to low-income people (Fleming and Freyhold, 2007). To date an economically viable and safe method of synthesis, capable of substituting extraction from the plant drug, has not been developed (Delabays et al., 2001; Fleming and Freyhold, 2007). The multivariate optimization has been increasing the quality of several products in pharmaceutical field. In this context, the ICH guidelines provide many points to get "quality by design" (ICH, 2009). Thus, the obtaining dry products with high levels of artemisinin have a great importance to apply the tools of the experimental design.

The hydroalcoholic extract of *A. annua* which has shown comparable antimalarial activity (Wright et al., 2010; Diawara et al., 2012) could be an alternative to the purified compound. The use of the vegetable drug with 0.23% of artemisinin has reported an IC_{50} value of 2.85 μg/ml for the hydroalcoholic extract, which is close to that of pure artemisinin, 2.73 μg/ml (Diawara et al., 2012). Thus, cultivars with a higher content of this compound could result in more interesting extracts in this respect.

The use of experimental design for assessing and optimizing extraction processes makes it possible to obtain maximum useful information, through fewer experiments, and thereby minimize

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costs and maximize desired responses. Response surface methodology (RSM) is a useful statistical technique for the construction of an empirical model, employing the most important variables and their effects (Erbay and Icier, 2009; Yang et al., 2009). Besides, was not found in the literature papers which investigated how to increase the artemisinin content by ultrasound assisted extraction from the leaves of *A. annua*.

The objective of this study was to establish the combination of parameters, ethanol graduation, previous shaking time in an ultrasound bath and drug/solvent ratio which would optimize artemisinin extraction efficiency from *A. annua* and then validate the method used. These three factors were selected because represent parameters with easy handling and are the main conditions investigated in several works found in literature. Thus, these variables might affect the yield of the artemisinin obtain by ultrasound assisted extraction.

Materials and methods

Materials

The reference standard artemisinin (98%) was purchased from Sigma Aldrich.

Aerial parts of *Artemisia annua* L., Asteraceae, were cultivated, dried and kindly provided by Divisão de Agrotecnologia do Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA) at the Universidade de Campinas, Brazil, in March 12, 2012 (block F1). Identification of the botanical material was done by Pedro Melillo de Magalhães (CPQBA).

For the extraction process, the dried plant was pulverized in a crusher with a helix and then particle size classification was carried out using a BecTel granulometer. The volatile content was determined on the Ohaus MB35 infrared balance. Weight measurements were performed on a RADWAG XA110 analytical balance.

Extraction procedure

Extraction was carried out by three stage re-maceration performed in a percolator vessel using 1000 ml percolators. Factors such as drug/solvent ratio (DSR), ethanol graduation (ETG) and previous shaking time in an ultrasound bath (PTU) were evaluated. Each factor was evaluated in triplicate at low, medium and high levels (−1, 1, and 0) with a medium point, yielding a total of fifteen experiments (Table 1). The factors were coded, following the given equation:

$$\text{Coded value} = \frac{\text{actual value} - 0.5 \times (\text{high value} + \text{low value})}{0.5 \times (\text{high value} - \text{low value})}$$

Table 1
Box–Behnken design for percolation of *Artemisia annua*.

Run	PTU (min)	DSR (%)	ETG (%)
1	−1 (0)	−1 (2)	0 (80)
2	1 (30)	−1 (2)	0 (80)
3	−1 (0)	1 (10)	0 (80)
4	1 (30)	1 (10)	0 (80)
5	−1 (0)	0 (6)	−1 (65)
6	1 (30)	0 (6)	−1 (65)
7	−1 (0)	0 (6)	1 (95)
8	1 (30)	0 (6)	1 (95)
9	0 (15)	−1 (2)	−1 (65)
10	0 (15)	1 (10)	−1 (65)
11	0 (15)	−1 (2)	1 (95)
12	0 (15)	1 (10)	1 (95)
13	0 (15)	0 (6)	0 (80)
14	0 (15)	0 (6)	0 (80)
15	0 (15)	0 (6)	0 (80)

The DSR factor was evaluated at levels of 2, 6 and 10% of the powdered drug; ETG was evaluated at levels of 65, 80 and 95%; and PTU was evaluated in a USC 4800 Unique® Ultra Cleaner ultrasonic washer (40 kHz) at levels of 0, 15 and 30 min.

The solvent (250 ml) was divided into three equal parts. One part was added to the calculated quantity of the plant drug in a glass beaker and submitted to ultrasound for a specified time. Each part of the solvent remained in contact with the plant drug for 24 h. After which time the percolator was opened, the extract transferred to a lidded container and a new part of fresh solvent added, giving an overall total of 72 h (3 × 24 h) of static maceration. The three parts of each condition were then brought together, homogenized, and an artemisinin assay was performed in each condition of the planning.

During three stage re-maceration, the statistical analysis was performed using Action® 2.7 software installed in Excel® (Office® 2010), with which the effect and interaction graphics, the model equation and its lack of fit were determined, with a 95% confidence interval.

Equipment and conditions

The artemisinin assay method was performed using a Waters® high performance liquid chromatography apparatus (HPLC), with e2695 separation module, a Waters® 2998 ultraviolet diode array detector equipped with Empower2® Build 2154 software. The Zorbax column (Agilent) Eclipse C18 (5 μm) 150 × 4.6 mm was protected by pre-column Phenomenex Security Guard C18 and maintained at 30 °C. The reading was taken at 255 nm and the mobile phase flow (1.2 ml/min) acetonitrile:0.2% formic acid (v/v) followed a gradient of 35:65 for 8 min, then changed to 60:40 for 5 min, returning to 35:65 and ending with a 20 min run.

Sample and standard solution preparation and derivatization

The vegetable drug was transferred to a volumetric flask and subjected to shaking for 40 min with 95% ethanol in an ultrasound bath. Then the volume was completed with the same solvent, homogenized, decanted and filtered through qualitative filter paper. An aliquot of 1 ml of hydroalcoholic extract was filtered and transferred to a 10 ml volumetric flask. Then 4 ml of 0.2% sodium hydroxide were added and, after 50 min the volume was completed with 0.2 M acetic acid, converting the artemisinin to a Q260 compound (Zhao and Zeng, 1986). The final solution was homogenized, and injected into the chromatographic system after 40 min.

The standard artemisinin was dissolved in 95% ethyl alcohol and prepared in the same way.

Method validation

The analytical method to assay artemisinin was validated on selectivity, linearity and range, precision, accuracy, limits of quantitation, detection and robustness parameters.

Selectivity was evaluated by comparing the retention time of the peak of interest in the sample with the peak obtained with standard artemisinin. An ultraviolet scanning at 190–400 nm was carried out to check the purity of the peak.

Areas versus concentration curves of the standard and plant drug were constructed for analysis of linearity. The linear correlation coefficient (r), adjusted determination coefficient (r^2) and the linear equation $y = ax + b$ were calculated, and statistical tests were done using Action® 2.7 software, installed in Excel® (Office® 2010). The significance of regression (ANOVA), lack of fit, normality of residuals using the Anderson–Darling method and homoscedasticity of the residuals using the Breush-Pagan method tests were performed with a 95% significance level.

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