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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 three stage re-maceration 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.

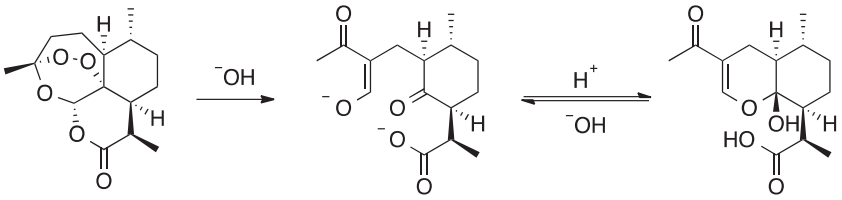
The standard artemisinin was dissolved in 95% ethyl alcohol and prepared in the same way.

### 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.



**Scheme 1.** Derivatization reaction of artemisinin (Zhao and Zeng, 1986).

Precision was verified on repeatability and intermediate precision levels. Accuracy was calculated using the standard addition method. To calculate the limits of quantification (LOQ) and of detection (LOD), three analytical curves were built with *A. annua* solutions at low concentrations, close to the supposed limits, using the formulae $LOD = 3S \times s^{-1}$, and $LOQ = 10S \times s^{-1}$, where $S$ is the standard deviation of the linear coefficients of the three calibration curves and $s$ is the mean of the slope of the three analytical curves. To analyze robustness, some parameters were varied in order to reproduce changes that could occur in routine analysis and interfere with the results, such as stability of the solution, wavelength, formic acid concentration in the mobile phase, column temperature and mobile phase flow.

System suitability parameters were calculated in peak of interest, such as Capacity Factor ($K$), Resolution ($R$), Tailing factor ($T$) and Theoretical Plates ($N$) (US-FDA, 1994) by Empower2® Build 2154 software.

## Results and discussion

### *Artemisinin derivatization*

The derivatization reaction of artemisinin yielded compound Q260, which absorbs about 60 times more artemisinin (Scheme 1). In reaction, through cleavage of the endoperoxide bridge, chromophore groups were formed in the Q260 compound (Zhao and Zeng, 1986).

This behavior occurred even in the derivatization performed using heating at 50 °C for 30 min, as previously described by other authors (Zhao and Zeng, 1986; Marchese et al., 2001; Hao et al., 2002; Erdemoglu et al., 2007; Diawara et al., 2011). Heating was not used in this experiment because it was not essential for the total hydrolysis of artemisinin and this reduced the area values of the chromatographic peaks by almost 15%.

### *Method validation*

Analytical method validation is an essential component of the measures carried out in a laboratory, which makes for the production of reliable results. The aim of validation is to show that the analytical method is suited to its purpose (Celeghini et al., 2009).

The system suitability parameters were within those recommended (US-FDA, 1994), which indicated that compound Q260 was satisfactorily separated from the other substances of the plant drug. Diawara et al. (2011) also performed the derivatization method with a satisfactory separation of the Q260 compound, with a retention time of 10.5 min.

The method was selective, and presented a compound Q260 retention time exactly the same for both the standard and *A. annua* (6.3 min) (Fig. 1). The scan spectrum of the peak in the vegetable drug chromatogram indicated that it was a single substance (Fig. 2), thereby showing that the separation was efficient.

The analytical curve of standard artemisinin resulted in an $r^2$ of 0.9996, with a concentration ranging from 247.9 to 743.8 μg/ml

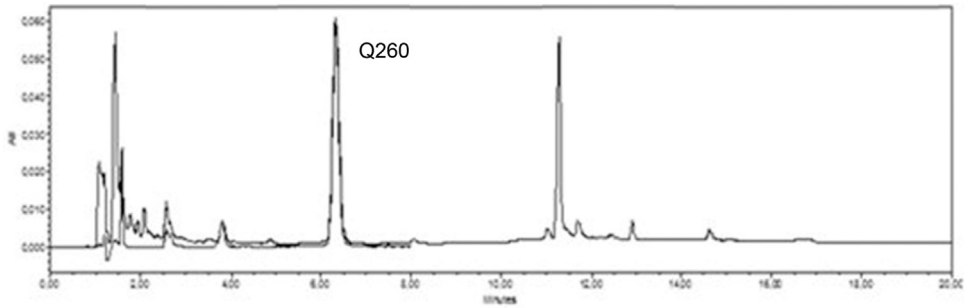



**Fig. 1.** Overlapping chromatograms of the compound Q260 for standard and *A. annua*.

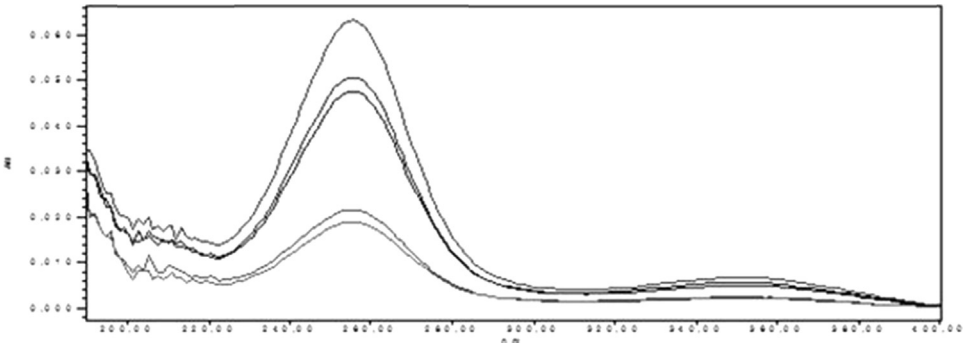


**Fig. 2.** Scan spectrum of the peak in the compound Q260 from *A. annua*.

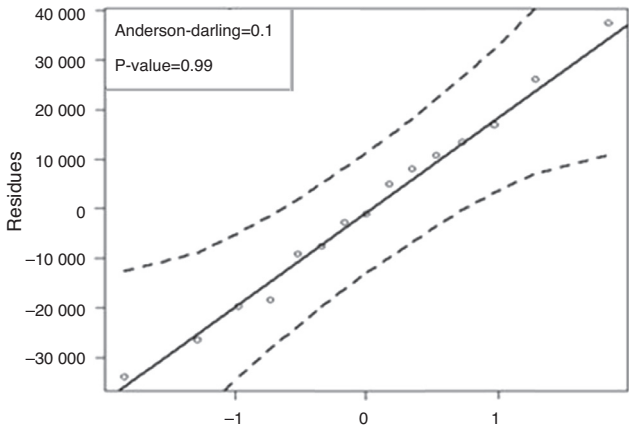



**Fig. 3.** Residue normality graph according to the Anderson–Darling test.

and a linear equation of $y = 12{,}277x - 25{,}352.4$. Analysis of the linearity of the plant drug resulted in an $r^2$ of 0.9925, with a concentration ranging from 25 to 75 mg/ml and a linear equation of $y = 13{,}373.7x - 39{,}053.9$. The residue normality graph is plotted in Fig. 3. The ANOVA test showed significance for linear regression ($p = 2.1E - 15$) and the model showed no lack of fit of the analytical curve ($p = 0.78$). For residues, the Anderson–Darling test indicated the presence of normal distribution ($p = 0.99$) and the Breush-Pagan test indicated homoscedasticity, *i.e.*, constant error variance ($p = 0.22$) between the points analyzed.

Some studies on method validation for the analysis of artemisinin in *A. annua* only describe the parameters obtained in the standard analysis of this compound and not the plant drug (Marchese et al., 2001; Erdemoglu et al., 2007; Liu et al., 2007; Celeghini et al., 2009). The standard presents a purity of more than 98%, while the vegetable drug usually presents between 0.01 and 1.5% of artemisinin (Atemnkeng et al., 2009). As other substances in the complex matrix of the plant drug can interfere in the results of the analytical method, it is therefore important to evaluate the validation parameters in the analysis of the plant drug.

Precision assessed for the vegetable drug at the repeatability level resulted in a relative standard deviation (RSD) of 3%, while at the intermediate level, the RSD was 4.5%. These values were lower than those obtained in other studies (Rehder et al., 2002; Diawara et al., 2011; Suberu et al., 2013). The accuracy of this method was 100.1 ± 4.5%, a satisfactory result when compared to the 102.3 ± 16.8% obtained by Diawara et al. (2011), while the standard deviation was over 3.7 times lower. The limits of detection and quantification were 1.3 and 4.0 μg/ml of artemisinin, respectively.

The method was robust for all parameters evaluated, with RSD values between 0.15 and 2.39% (Table 2). The values of peak areas related to the compound Q260 were stabilized only at least 40 min after the addition of 0.2 M acetic acid at the end of derivatization, which increased the precision and accuracy of the method.

**Table 2**
Results of the robustness test analysis.

| Parameters evaluated | Altered condition | RSD (%) |
|---|---|---|
| Solution stability | 0–210 min | 0.58 |
| Wavelength | ±2 nm | 0.29 and 1.22 |
| Formic acid concentration in the mobile phase | ±10% (v/v) | 0.33 and 0.67 |
| Column temperature | ±2 °C | 0.15 and 0.45 |
| Flow rate | ±0.03 ml/min | 0.85 and 2.39 |

**Table 3**
Artemisinin content in each assay of extraction by three stage re-maceration.

| *n* | Parameters | | | Artemisinin % (m/m) |
|---|---|---|---|---|
| | PTU (min) | DSR (%, m/v) | ETG (%, v/v) | |
| 1 | 0 | 2 | 80 | 1.08 |
| 2 | 30 | 2 | 80 | 1.02 |
| 3 | 0 | 10 | 80 | 0.92 |
| 4 | 30 | 10 | 80 | 0.94 |
| 5 | 0 | 6 | 65 | 1.05 |
| 6 | 30 | 6 | 65 | 0.99 |
| 7 | 0 | 6 | 95 | 1.02 |
| 8 | 30 | 6 | 95 | 0.96 |
| 9 | 15 | 2 | 65 | 1.08 |
| 10 | 15 | 10 | 65 | 0.90 |
| 11 | 15 | 2 | 95 | 1.21 |
| 12 | 15 | 10 | 95 | 0.92 |
| 13 | 15 | 6 | 80 | 0.98 |
| 14 | 15 | 6 | 80 | 0.97 |
| 15 | 15 | 6 | 80 | 0.98 |

*Extraction procedure*

The *A. annua* used in this experiment came from the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA) at the Universidade de Campinas (Unicamp, Brazil) and contained about 1.2% artemisinin (Rodrigues et al., 2006; Celeghini et al., 2009).

Maceration and percolation are extraction methods in which different solvents are used to obtain artemisinin (Briars and Paniwnyk, 2013). Fleming and Freyhold (2007) used ethanol as an extractor liquid and obtained the best results with ethanol content of more than 90%. They further concluded that ethanol extracts more substances, which could be advantageous when the aim is to obtain artemisinin extract and the other compounds present in the synergistic plant drug.

The amount of the compound of interest extracted for each condition is presented in Table 3. The maximum artemisinin content of 1.21% is similar to that obtained for *A. annua* from the CPQBA (Rodrigues et al., 2006; Celeghini et al., 2009).

The Pareto graph (Fig. 4) showed that increased DSR was the main factor which influenced extraction efficiency, and reduced linear efficiency, as found by Fleming and Freyhold (2007). The regression equation that represents the model for artemisinin content is shown in Eq. (1) and $R^2$ adj = 0.9888. This result of $R^2$ adj demonstrates that the model is highly explainable by analyzed variables.

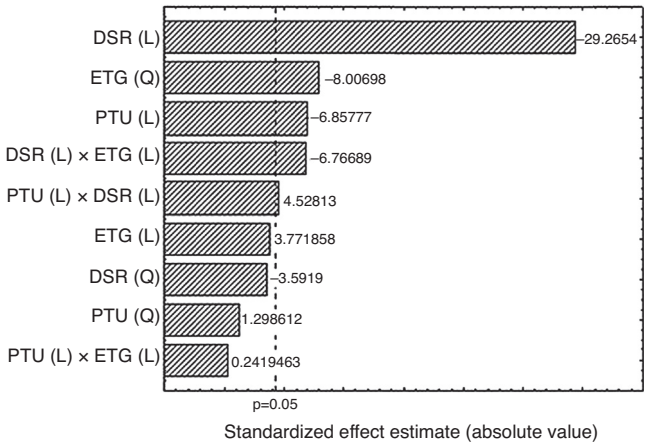



**Fig. 4.** Pareto graph in relation to artemisinin extraction.

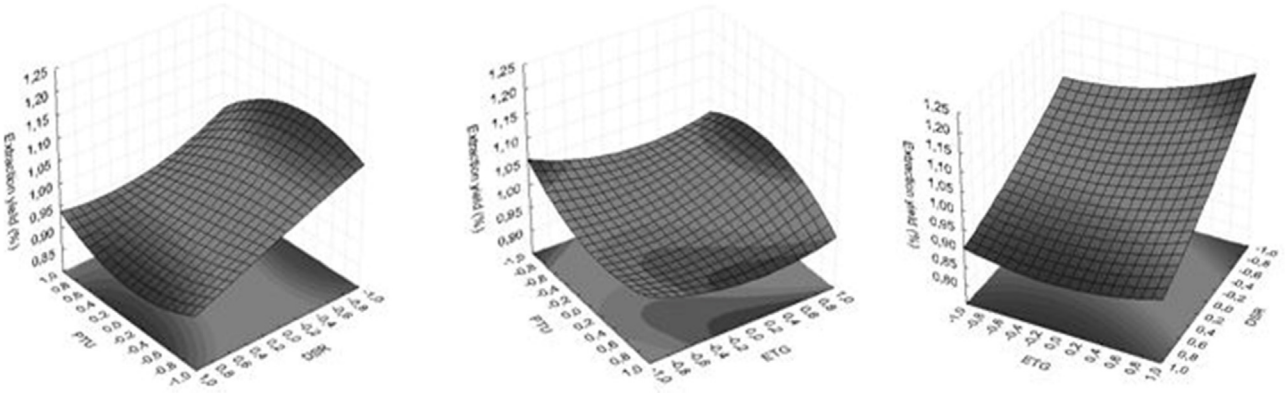



**Fig. 5.** Response surface graph of artemisinin yield with PTU × DSR (left), PTU × ETG (center) and ETG × DSR (right).

$$\text{Art} = 0.98 - 0.030A - 0.12B + 0.037C + 0.020AB + 0.001AC - 0.027BC - 4.583E - 003A^2 + 0.018B^2 + 0.033 * C^2 + + 0.057A^2B - 0.052A^2C + 0.020AB^2 \quad (1)$$

Response surface methodology (RSM) is a statistical technique which is used for the construction of an empirical model, employing the most important variables and their effects (Erbay and Icier, 2009; Yang et al., 2009). The Box–Behnken experimental design, based on a fractional factorial design at three levels (low, medium and high) is used for each factor studied (Portal Action, 2014).

From the gradient model, the optimal conditions (Fig. 5) for the best extraction efficiency occurred with a PTU of 12.28 min, a DSR of 2.03% and an ETG of 94.96%.

It was not found in the literature papers that apply the design of experiments (DOE) in the extraction of *A. annua* extraction.

## Conclusion

From the Box–Behnken experimental design, the best combination of the DSR, ETG and PTU factors was found. The main factor underlying artemisinin extraction efficiency was the DSR. Information obtained in this study can be used for future approaches to determining and extracting artemisinin from *A. annua*. Validation of the analytical method for determining artemisinin from *A. annua* complied with all the specifications of the tests. Using derivatization of the compound, the HPLC-UV method increased detector response and made it possible to increase the limits of detection and quantification.

## Authors contributions

EOS: acquisition of the data, analysis and interpretation of the data, drafting of the article; LLB: statistical expertise, drafting of the article; ECC: conception and design, provision of study materials; MTFB: conception and design, provision of study materials, obtaining of funding, drafting of the article.

## Conflicts of interest

The authors declare no conflicts of interest.

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