

Optimization of Ultrasound-assisted extraction of polyphenols, tannins and epigallocatechin gallate from barks of *Stryphnodendron adstringens* (Mart.) Coville bark extracts

Jordana N. Sousa, Nathália B. Pedroso, Leonardo L. Borges, Gerlon A. R. Oliveira, José R. Paula, Edemilson C. Conceição

Faculty of Pharmacy, Federal University of Goiás, Goiás, Brazil

Submitted: 09-08-2013

Revised: 22-10-2013

Published: 28-05-2014

ABSTRACT

Background: *Stryphnodendron adstringens* (Mar.) Coville is a native plant from Brazil, rich in phenolic compounds and used on popular medicine as a wound healing agent, in the treatment of gastric lesions and as antimicrobial. **Materials and Methods:** Ultrasound-assisted extraction (UAE) was applied to extraction of epigallocatechin gallate (EGCG), total polyphenols (TP) and total tannins (TT) content from barks of *Stryphnodendron adstringens* (Mar.) Coville. Several operating parameters, namely extraction time (min), liquid to solid ratio (mg/mL), ethanolic strength (% v/v), were optimized using response surface methodology (RSM) with a Box-Behnken design. **Results:** By using the desirability function approach, the optimum UAE conditions to obtain desirable extraction yields for all these metabolites simultaneously were found at the extraction time of 30 min, solid to liquid ratio of 4 mg/mL and ethanolic strength of 65. Under these conditions, the epigallocatechin gallate, total polyphenols and total tannins content were 0.31; 22.95 and 11.95 % (w/w), respectively. **Conclusion:** The results indicated that knowledge gained from this study should be helpful to further exploit and apply this resource and also showed the feasibility of ultrasound-assisted extraction for obtaining EGCG, TP and TT from barks of *S. adstringens*.

Key words: Desirability function, optimization, response surface methodology, *Stryphnodendron adstringens*, ultrasound-assisted extraction

Access this article online

Website:

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DOI:

10.4103/0973-1296.133287

Quick Response Code:



INTRODUCTION

Stryphnodendron adstringens (Mar.) Coville is a native plant from Brazilian Savannah popularly known as barbatimão.^[1] Many therapeutic properties are attributed to *S. adstringens* as gastric antiulcerogenic,^[2,3] trypanocidal,^[4] antimicrobial,^[5-7] antioxidant^[8,9] and as wound healing.^[10]

Medicinal properties are related to high phenolics compounds as tannins in the barks, at least 8%.^[11] Flavan-3-ols, prodelphinidins and prorobinetinidins were isolated and identified.^[12] Between flavan-3-ols identified, epigallocatechin-3-O-gallate (EGCG) stands out for being an antioxidant with antimutagenic and anticarcinogenic activities.^[13]

Recently, the use of *S. adstringens* in the production of an herbal medicine with anti-inflammatory and wound healing were related.^[14] In addition, there are 16 patent applications filed in National Institute of Industrial Property (INPI) about products containing *S. adstringens* with various applications.^[15] However, to ensure safety, efficacy and quality of medicines regulatory agencies as FDA (Food and Drug Administration) and Agência Nacional de Vigilância Sanitária (ANVISA - Brazilian Health Surveillance Agency) require that herbal extracts are standardized.^[11,16] This standardization occur through pharmacognostic characterization, identification of chemical composition by various chromatographic techniques and biological activity of the whole plant.^[17,18]

One of the key steps in phytopharmaceutical technology studies is the extraction method that will be used to extract the maximum particular constituent or group

Address for correspondence:

Prof. Jordana Nunes de Sousa, Rua gr2 qd 10 lt 17 St. Grande Retiro, Goiânia, Goiás, Brazil.
E-mail: jordanans88@gmail.com

from the plant raw material, leaving as residue the inert or undesirable.^[19] (HELOU, 1989)(Helou, 1989) In cases such as this, where many factors influence the response of the system, optimization of the extraction procedure can be carried out using multivariate statistical tools.^[20] Among the most relevant multivariate techniques used in analytical optimization, response surface methodology (RSM) allows the evaluation of the effect of multiple variables and their interactions on the variables output with reduced number of attempts, which aims improve the performance of the systems and increase the yield of the processes without increasing the cost.^[21,22] (BAŞ; BOYACI, 2007; WU *et al.*, 2007)(Baş and Boyacı, 2007; Wu, Cui, Tang, and Gu, 2007) Aiming to approximate a response function to experimental data that cannot be described by linear functions, experimental designs for quadratic response surfaces should be used, such as three-level factorial, central composite, Doehlert designs and Box–Behnken.^[23]

Despite the therapeutic potential of *S. adstringens* and its popular use and industrial pharmaceuticals importance, there are no data about supply an optimal method to extract secondary metabolites for quality control of raw material or biological assays. The aim of this work was the optimization of ultrasound-assisted extraction procedure for determining epigallocatechin gallate (EGCG), total polyphenols (TP) and total tannins (TT) content from barks of *S. adstringens*. To achieve this were investigated extraction time, solid to liquid ratio and ethanolic strength influence at UAE. Experimental design combined with desirability functions was applied for optimization purposes.

MATERIALS AND METHODS

Chemicals

EGCG (95%) and tannic acid (98%) were purchased from Sigma-Aldrich® (Sigma-Aldrich Brasil Ltda, São Paulo, Brazil). Acetonitrile was analytical grade (Merck KGaA, Darmstadt, Germany). Additionally, phosphoric acid (Merck KGaA, Darmstadt, Germany), and ultrapure water from a Milli-Q system (Millipore®, Bedford, MA, USA) were used.

Herbal material

The plant material was bought at Paladar condimentos company ® (Brazil, Goiás) and their authenticity was confirmed by Dr. José R. Paula. The sample was cleaned and dried in an oven (Solab Scientific ®) with forced circulation and air renewal at 40°C for 48 h. After dehydration, the dried plant material was ground in a knife mill TE-625 (Tecnal Ltda, Piracicaba, SP, Brazil) and stored sheltered from light and moisture. The pharmacognostic characterization of plant material revealed moisture content of $8.74 \pm 0.09\%$, total ash of $1.45 \pm 0.04\%$ and particle size of $344.50 \mu\text{m}$.^[24]

Total polyphenol contents (TPc) and total tannin contents (TTc)

TPc and TTc were determined following previously described methods.^[25,26] Solid to liquid ratio (4, 6, 8 mg/mL) were diluted in hidroalcoholic solution (65, 80 and 95%) in both TPc and TTc measurements Table 2. The standards curves were prepared with tannic acid, at the dilutions: 100, 200, 300, 400, 500 and 600 $\mu\text{g/mL}$.

Instrumentation and chromatographic conditions

The presence of EGCG was confirmed by TLC (thin layer chromatographic) on silica gel 60 F254 layers using etil acetate:toluene:formic acid:water (80:10:5:5 *v/v/v/v*) as mobile phase. (Brazilian pharmacopeia, 1988) The TLC analysis of the EGCG compound at sample revealed a retention factor of 0.57 as observed for the standard EGCG. In the HPLC method, EGCG was identified and quantified using a HPLC Waters® e2695 (Milford, Massachusetts, USA), comprising a quaternary pump, an on-line degasser, an autosampler and a photodiode array detector model 2998. The treatment of data and control of HPLC equipment were performed in Empower® 2.0 software. A gradient elution was performed on an Agilent Technologies® (USA) C18 (250 mm × 46 mm × 5 μm). The presence of EGCG was confirmed by comparison of

Table 1: Code and level of factors chosen for the trials

Factor	Low	Center	High
Extraction time (v/v%, ES)	-1 (15)	0 (30)	1 (45)
Solid to liquid ratio (mg/mL, SLR)	-1 (4)	0 (6)	1 (8)
Ethanol strength (v/v%, ES)	-1 (65)	0 (80)	1 (95)

SLR: Solid liquid ratio; ES: Ethanol strength

Table 2: Experimental results

Run	ET	SLR	ES	EGCG	PTc	TPPc
1	-1	-1	0	0.27	17.40	11.10
2	1	-1	0	0.31	19.28	10.62
3	-1	1	0	0.23	13.06	8.20
4	1	1	0	0.31	13.56	7.65
5	-1	0	-1	0.28	18.08	10.42
6	1	0	-1	0.31	14.32	10.65
7	-1	0	1	0.17	15.09	9.43
8	1	0	1	0.18	12.55	7.12
9	0	-1	-1	0.30	22.60	11.35
10	0	1	-1	0.30	10.05	7.38
11	0	-1	1	0.18	27.29	10.68
12	0	1	1	0.19	13.26	8.39
13	0	0	0	0.30	16.21	9.14
14	0	0	0	0.28	17.11	9.67
15	0	0	0	0.29	17.11	9.40

Xi: Coded factors in the experimental design; -1, 0, 1: Coded levels in the experimental design, according to Table 1 Et: Extraction time (h); SLR: Solid to liquid ratio (-); ES: Ethanolic strength (%); EGCG: Epigallocatechin gallate contents (% w/w d.b.); TP: Total polyphenol contents (% w/w d.b.); TPPc: Tannins by precipitation of proteins contents (% w/w d.b.)

their retention time, spiking the extract with standard and overlaying UV spectra with standard compound at a 208 nm.

The mobile phase was 0.1% (*v/v*) orthophosphoric acid in water (A) and acetonitrile (*v/v*) (B) at a flow rate of 1 mL/min at 30°C. The program was set at: 0 min to 10 min: 90% (A), 18 min: 75% (A), 22 min: 50% (A), 26 to 30 min 90% (A). All solutions were degassed and filtered through a 0.45 µm pore size filter (Millipore, USA).

The method presented linearity over the range from 0.002 to 0.024 mg/mL ($R^2 = 0.995$); limit of detection and limit of quantification of 0.09 µg/mL and 0.3 µg/mL, respectively; suitable selectivity; accuracy of 99.06%; within-and between day precisions with relative standard deviation values of 2.46 and 2.70%, respectively.

Design of experiments

Box-Behnken experimental design (3^3) with three factors and three levels was used to optimize and evaluate main effects, interaction effects and quadratic effects of the process variables.^[27] The factors studied were: extraction time (min, *ET*), liquid to solid ratio (mg/mL, *SLR*) and the ethanolic strength (*v/v*%, *ES*) Table 1. Experiments were performed using a 40 kHz ultrasonic UNIQUE® (USC 4800).

The response function applied was a second-order polynomial regression model, given by Equation 1:

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum \sum \beta_{ij} x_i x_j \quad (1)$$

Where *y* represents the response variables, β_0 is a constant, β_i , β_{ii} and β_{ij} are the linear, quadratic and cross-product coefficients, respectively. Independent variables are represented by X_i and X_j .

Desirability function (DF) is a technique to determine of input variables that can give the optimum conditions for one or more responses. The procedure involves three steps: (1) predicting responses on the dependent variable by fitting the observed responses using an equation, based on the levels of the independent variables, (2) finding the levels of the independent variables that simultaneously produce the most desirable predicted responses on the dependent variables and (3) maximize the overall desirability with respect to the controllable variables.^[28, 29] Therefore, the main advantages of using desirability functions are to obtain qualitative and quantitative responses by the simple and quick transformation of different responses for one measurement. Firstly, the response is converted into a particular desirability function that varies from 0 to 1. The individual desirability scores for the predicted values for each dependent variable

are then combined into overall desirability function by computing their geometric mean of different individual values. The desirability 1 is for maximum and desirability 0 is for non-desirable situations or minimum. All statistical analysis was conducted using the software Design-Expert® version 7.0.^[30] The factors with significance higher than 5% ($P < 0.05$) was considered. All experiments were conducted in triplicate.

RESULTS AND DISCUSSION

The results of UAE experiments are summarized in Table 2. Under the established conditions, EGCG values ranged from 0.17 to 0.31% (*w/w*), the TPc ranged from 10.05 to 27.29 % (*w/w*) and TPPc 7.12 to 11.35% (*w/w*).

The ANOVA Table 2 showed that *ET*, *ES* and ES^2 exerted an influence on the EGCG content at a significance level of 5%. None of ET^2 , *SLR*, SLR^2 nor the interactions was significant. Moreover, increasing *ET* and ES^2 had a positive influence on EGCG content; besides, there are a negative relationship between EGCG and *ES*. Figure 1 presents the surface responses of EGCG as a function of *ET* and *ES*. The fitted equation, with correlation coefficient $R = 0.9863$, is given by:

$$EGCG = 0.29 + 0.02ET - 0.058ES - 0.045ES^2$$

Lower values of *ES* and higher *ET* increase the extraction of EGCG as can be seen in Figure 1.

Figure 2 shows a surface plot of total phenols content (TPc) as a function of the *ET* and *SLR*. The surface shows that the *SLR* exerted a negative influence on TPc, which was confirmed by ANOVA at level of 5% of significance. The surface also shows that *ET* exerted a negative nonlinear effect on TPc, with the higher values of TPc around the level 0 of extraction time due the quadratic behavior of this model, indicating that the optimum point is close of this region. The fitted equation presented correlation coefficient $R = 0.8682$.

$$TPc = 16.81 - 2.14ET^2 - 5.58SLR + 1.15SLR^2$$

In accordance to ANOVA, only the *SLR* exerted linear influence on the TPPc at a significance level of 5%. None of *ET*, ET^2 , SLR^2 , *ES*, ES^2 nor the interactions was significant. Moreover, increasing the *SLR* had a negative influence on total tannins content. The fitted equation, with correlation coefficient $R = 0.9151$, is given by:

$$TPc = 9.91 - 1.52SLR.$$

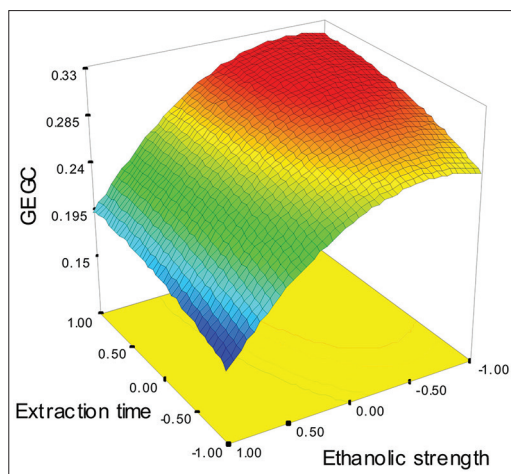


Figure 1: Response surface of EGCG as a function of extraction time (ET) and ethanolic strength (ES)

Effect of extraction parameters on different phenolic compounds

The progressive addition of extraction time improves the EGCG and TP contents. This results are in good agreement with.^[31] The UAE facilitated the swelling and hydration causing the enlargement in the pores of the cell wall, this will contribute to diffusion process and therefore the enhancing mass transfer, so the major extraction time contributes to metabolites extraction.^[32]

The increased solid to liquid ratio showed negative TPc and TTc Table 2, which probably reflects a tendency toward solvent saturation or, at least, a lessening in extraction effectiveness due to the high content of both.

A combination of alcohol with water is more effective in extracting EGCG compounds than alcohol only. Pure EGCG is soluble in water at least to 5 mg/mL, giving a clear faint yellow solution. HPLC analysis confirmed the decreasing of ethanolic strength was most effective, among solvents tested, for the prolonged extraction (30 min) of catechins, especially in the extraction of EGCG, the dominant catechin in tea leaves.^[33]

Extraction optimization

The models adequacies were checked accounting for Pearson's correlation values, which were higher than 0.80. This, together with the fact that the F values of fitted models were significant ($P < 0.05$), indicates that the regression models presented a great adjust, being suitable to predict the responses and explain their behavior.^[34]

In further analysis of these responses, optimization was carried out using Design Expert 7.0 to obtain the criteria for maximum EGCG, TPc and TPPc. By applying a desirability function method, a total of 13 conditions were found to satisfy the goal, all with good desirability values, the ET

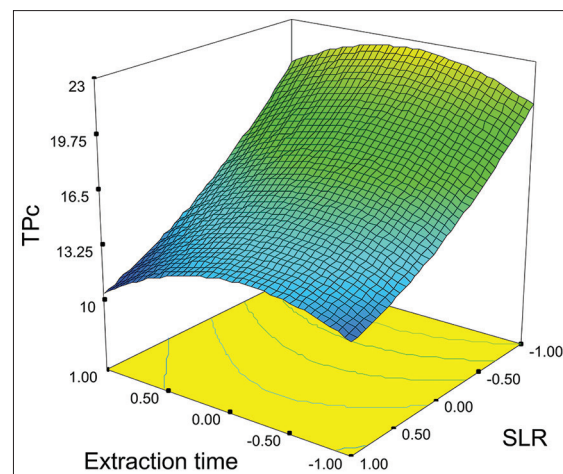


Figure 2: Response surface of TPc as a function of extraction time (ET) and solid liquid ratio (SLR)

Table 3: Summary of factor effects on powder properties

Factor	EGCG	TPc	TTc
Mean	0.253476	16.37762	9.41597
ET	0.040664*	-0.97635	-0.77756
ET ²	0.010747	2.13785*	0.28148
SLR	-0.007403	-9.16192*	-3.03201*
SLR ²	0.000371	-1.15211	0.23689
ES	-0.116836*	0.78414	-1.04548
ES ²	0.045294*	-0.34039	0.22281
ET x SLR	0.016619	-0.69120	-0.03755
ET x ES	-0.005497	0.60787	-1.26725
SLR x ES	0.005717	-0.73900	0.84249

*Significant effects ($P < 0.05$). ET: Extraction time; SLR: Solid to liquid ratio; ES: Ethanolic strength; EGCG: Epigallocatechin gallate contents; TPc: Total polyphenol contents; TTc: Total tannin contents

ranged from 27.3-32.85 min; solid to liquid ratio ranged of 4 mg/mL and ethanolic strength between 65 to 74.6%. At these conditions, the predicted yield was 0.31% (w/w) for EGCG, 21.95-22.31% (w/w) for TPc and 11.49-11.95% (w/w) for TTc, see Figure 3. To confirm the theoretical results, three parallel experiments were carried out under those theoretical conditions, among the 13 optimal conditions provide by desirability function method all conditions showed good values of desirability, extraction time close and the same solid to liquid ratio; so the economy of the method was directed to choose, were tested the conditions with high, medium and low ethanolic strength. The theoretical and experimental condition are shown in Tables 3 and 4 and the condition 3, besides being the most economical, also presented the best yields with all experimental values close to theoretical, 0.31% (w/w) to EGCG, 22.50 % (w/w) to TP and 11.95% (w/w) to TT of EGCG, TPc and TPPc 0.31, 22.50 and 11.95% (w/w). In conclusion, a new optimization method based on a combination of UAE and RSM was investigated for the extraction of EGCG, TPc and TPPc. The RSM method was based on a three-level, three-variable

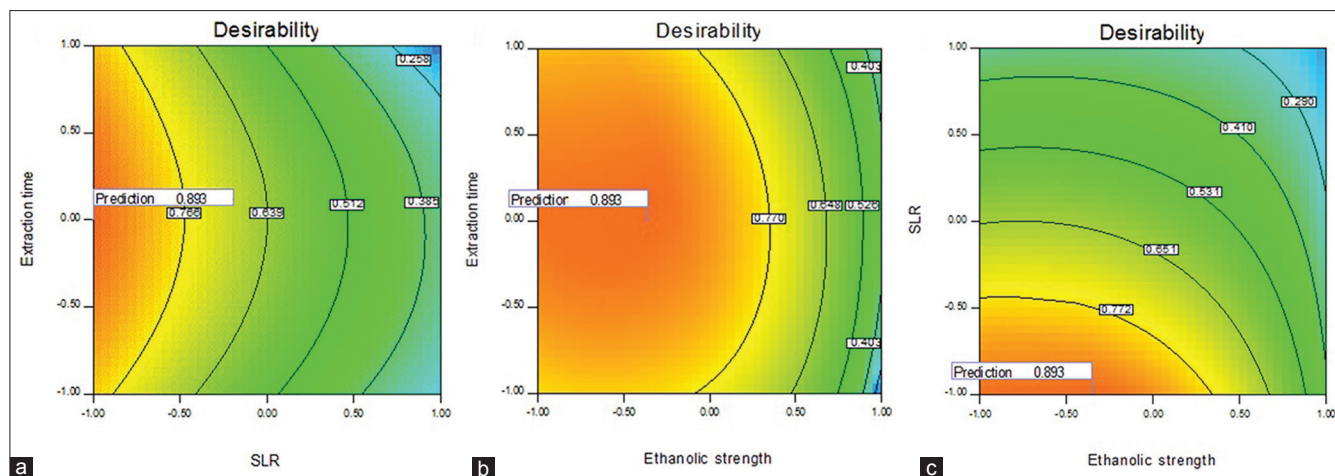


Figure 3: Countour plot of Desirability as a function of extraction time (ET) and solid to liquid ratio (SLR) (a); extraction time (ET) and ethanol strength (ES) (b); solid to liquid ratio (SLR) and ethanol strength (ES) (c)

Table 4: Theoretical and experimental conditions to validate the desirability model

Condition	D values	Optimized condition (modified experimental conditions)			Predicted yield (yield under modified conditions \pm SD)		
		ET (min)	SLR (mg/mL)	ES (%)	EGCG	TPc	TTc
1	0.892673	30 (30)	4 (4)	74.6 (75)	0.31 (0.29 \pm 0.5)	22.31 (22.00 \pm 0.6)	11.49 (10.90 \pm 1.1)
2	0.889592	27 min 27 s (27)	4 (4)	70.55 (70)	0.31 (0.30 \pm 0.9)	22.18 (21.90 \pm 0.28)	11.68 (11.21 \pm 1.4)
3	0.883893	30 (30)	4 (4)	65 (65)	0.31 (0.31 \pm 1.0)	22.95 (22.50 \pm 0.0)	11.95 (11.45 \pm 1.5)

SD: Standard deviation; ET: Extraction time; SLR: Solid to liquid ratio; ES: Ethanol strength; EGCG: Epigallocatechin gallate contents; TPc: Total polyphenol contents; TTc: Total tannin contents

(extraction time, solid to liquid ratio and ethanol strength extraction). The maximum extraction yield of EGCG, TPc and TPPc 0.31, 22.50 and 11.95% (w/w) was obtained by performing UAE with 65 % ethanol, in SLR of 4 mg/mL for 30 min. The results indicated that the UAE-RSM approach was effective for maximizing the extraction of EGCG, total phenol and tannins and the knowledge gained from this study should be useful for further exploitation and application of the resource.

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Cite this article as: Sousa JN, Pedroso NB, Borges LL, A. G, Paula JR, Conceicao EC. Optimization of Ultrasound-assisted extraction of polyphenols, tannins and epigallocatechin gallate from barks of *Stryphnodendron adstringens* (Mart.) Coville bark extracts. *Phcog Mag* 2014;10:318-23.

Source of Support: Nil, **Conflict of Interest:** None declared.