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Optimization of ultrasound-assisted extraction of phenolic compounds and flavonoids from *Hortia oreadica* leaf

[Optimización de la extracción asistida por ultrasonido de compuestos fenólicos y flavonoides de la hoja de *Hortia oreadica*]

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Abstract: *Hortia oreadica* is indiscriminated used by people from Cerrado. However, vegetable raw material quality is decisive in obtaining intermediate and final products. So, this study aimed to establish quality parameters of *H. oreadica*. For this, we performed the phytochemical screening of *H. oreadica* leaf and identified the best extractive conditions for phenolic compounds and flavonoids using factorial experimental design, varying the alcoholic strength, extraction temperature, and solid/liquid ratio in the ultrasound-assisted extraction method. The optimum extraction condition for phenolic compounds and flavonoids was 60% alcoholic strength, 40°C temperature, and a solid/liquid ratio of 8 mg/mL. Under this setting, the phenolic and flavonoid contents were 0.171 ± 0.002 mg/mL (predicted value = 0.165) and 0.087 ± 0.002 mg/mL (predicted value = 0.084), respectively. The optimized extraction parameters could be upscaled to develop pharmaceutical drugs or nutraceutical products from this non-traditional plant species using an eco-friendly approach.

Keywords: Central composite rotatable design; Cerrado; Quina-do-campo; Medicinal plants; Rutaceae.

Resumen: *Hortia oreadica* es utilizada indiscriminadamente por la gente del Cerrado. Sin embargo, la calidad de la materia prima vegetal es determinante en la obtención de productos intermedios y finales. Por lo tanto, este estudio tuvo como objetivo establecer parámetros de calidad de *H. oreadica*. Para ello, realizamos el tamizaje fitoquímico de la hoja de *H. oreadica* e identificamos las mejores condiciones extractivas para compuestos fenólicos y flavonoides mediante un diseño experimental factorial, variando el grado alcohólico, la temperatura de extracción y la relación sólido/líquido en el método de extracción asistido por ultrasonido. La condición óptima de extracción para compuestos fenólicos y flavonoides fue de 60% de grado alcohólico, 40°C de temperatura y una relación sólido/líquido de 8 mg/mL. Bajo esta configuración, los contenidos de fenoles y flavonoides fueron 0,171 ± 0,002 mg/mL (valor previsto = 0,165) y 0,087 ± 0,002 mg/mL (valor previsto = 0,084), respectivamente. Los parámetros de extracción optimizados podrían ampliarse para desarrollar fármacos o productos nutracéuticos a partir de esta especie de planta no tradicional utilizando un enfoque ecológico.

Palabras clave: Diseño giratorio compuesto central; Cerrado; Quina-do-campo; Plantas medicinales; Rutaceae.

INTRODUCTION

Hortia oreadica Groppo, Kallunki & Pirani (Rutaceae) is a Cerrado shrub with a subterranean stem well developed that confers resistance to arid climate during the dry season, fire protection, and good production of vegetable mass throughout the year (Groppo & Pirani, 2005). *H. oreadica*, known as "quina" (Saint-Hilaire, 1824), "quina-do-campo" (Pio-Corrêa, 1984), and "para-tudo" (Groppo & Pirani, 2012), is popularly used as antipyretic (Saint-Hilaire, 1824), as a substitute for quinine (Cinchona - Rubiaceae) (Pio-Corrêa, 1984), to treat stomach disorders, fever, diarrhea, vomiting, and liver diseases (Pio-Corrêa, 1984; Groppo & Pirani, 2012). Phenolic compounds extracted from *H. oreadica* presented antimicrobial (Melliou *et al.*, 2005; Severino *et al.*, 2015), cytotoxic (Cazal *et al.*, 2013), spasmolytic (Abbaskhan *et al.*, 2012), hepato-protective (Atmaca *et al.*, 2011), antiulcerogenic (Choi *et al.*, 2012), insecticidal (Mukandiwa *et al.*, 2013; Mukandiwa *et al.*, 2015), and antiparasitic (Severino *et al.*, 2009) potential, corroborating the popular use.

As plant species can undergo qualitative and quantitative variations throughout the year, it is essential to establish quality parameters for those with therapeutic and technological potential. Quantifying chemical markers in the vegetable raw material is also helpful in setting quality parameters. For phenols and flavonoids extraction, the ultrasound-assisted extraction procedure is one of the most applied because it is simple and uses small amounts of raw material, with reduced extraction time and solvent consumption, being considered eco-friendly (Azwanida, 2015; Braga *et al.*, 2017).

To optimize the chemical markers extraction in a solid-liquid phase, the response surface methodology (RSM), proposed by Box and Wilson (1951), has been widely used as a statistical approach. Central composite rotatable design (CCRD) consists of one of the most used experimental designs for this purpose due to its efficiency and flexibility. In addition, simultaneous optimization of multiple responses and their interactions is performed using desirable functions (Hossain *et al.*, 2011). So, fewer experimental runs are needed to optimize a reaction, a great benefit when an industrial process is taken into account (Yusof *et al.*, 2021).

In this sense, this study aimed to establish quality parameters of *H. oreadica* leaf. For this, we performed the phytochemical screening of *H. oreadica* leaf; and optimized an ultrasound-assisted extraction of total phenols and flavonoids using a CCRD approach, varying the alcoholic strength, extraction temperature, and solid/liquid ratio, to obtain the best extractive condition.

MATERIAL AND METHODS

Plant material

The *H. oreadica* leaf were collected in Pirenópolis, Goiás (15°48'15" S, 48°52'48" W, 1295 m). A voucher specimen was identified by Prof. Dr. Heleno Dias Ferreira and deposited in the Herbarium of UFG under the code number UFG-47798. Leaves were oven-dried at 40°C, powdered in a knife mill, and packed in a closed container.

Physicochemical parameters

The content of volatile compounds was determined in a humidity analyzer that produces radiation in the infrared region employing a halogen lamp (Gibertini model Eurotherm) (Brasil, 2010a). For this, 1 g of the leaf powder was weighed, distributed in the collector, and the tub was heated to 105°C until constant weight. The tests were performed in triplicate, and the mean, standard deviation, and coefficient of variation were calculated. The total ash and acid insoluble ash contents were performed according to the Brazilian Pharmacopoeia (Brasil, 2010a). All assays were performed in triplicate. The granulometric distribution was performed according to Brazilian Pharmacopoeia (Brasil, 2010a) using tamis with 1000 µm, 850 µm, 425 µm, 250 µm, 150 µm, and 125 µm mesh. The same was done to obtain the intumescence index. The assays were performed in triplicate.

Phytochemical screening

Phytochemical screening of the *H. oreadica* leaf powder was performed using the methodologies adapted from Costa (2001a), Matos (2009), Matos & Matos (1989) for digital heterosides, flavonoids, saponins, tannins, alkaloids, and coumarins.

Quantitative analysis of total phenols (TP) and flavonoids (Fv), and optimization of the ultrasound-assisted extraction

The quantitative analysis for total phenols was performed by the Hagerman and Butler adapted method (Mole & Waterman, 1987). The method described by Rolim *et al*. (2005), was used for flavonoid content.

To optimize total phenols (TP) and flavonoids (Fv) extraction, a CCRD with six central points was conducted, evaluating the effect of three factors: temperature (°C) (X1), solid-liquid ratio (SLR, g/ml) (X2) and alcoholic strength (%) (X3) (Table No. 2) over the phenolic compounds and total flavonoids concentration, whereby 20 different combinations were obtained (Table No. 3). All experiment runs were executed in triplicates, and the response (denoted by Y) was presented as the average TP or Fv measured. Based on the data obtained from the experiments, a regression analysis was performed, and an empirical second-order polynomial model was fitted. The equation below shows the relationship:

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

Where Y = response (TP or Fv) and the regression coefficients are denoted by β_0 = intercept, β_i = linear, β_{ii} = quadratic, β_{ij} = interaction, whereas x_i and x_j = independent variables.

The desirability function approach evaluated the optimized values for the independent variables. Derringer's approach involves first converting each response, y_k, into individual desirability, d_k. The desirability scale ranges from 0 to 1. If the response is outside an acceptable region, d_k is set to 0; if the response is fully desirable, d_k is set to 1. Considering the responses with significant effects on the experimental design, the d_k value is expected to be closer to 1. The individual desirability functions from the analyzed responses are then combined to obtain the overall desirability *D*, which is the geometric average of the individual desirability values, as represented below.

$$D = (d_1, d_2, \ldots, d_k)^{1/k}$$

The D value ranges from 0 to 1, and a high value of D is considered the best solution for the system (Hu *et al.*, 2008).

Data were analyzed using Design Expert 7.0 software (Stat-Ease, Inc., Minneapolis, MN).

Table No. 2
Evaluated factors and their levels used in the central composite rotatable design (CCRD) applied to TF and Fv ultrasound-assisted extraction from Hortia oreadica leaves.

Independent variables	Levels				
	-1.68	**-1**	**0**	**+1**	**+1.68**
T (°C) (X_1)	37	40	45	50	53
SLR (mg/ml) (X_2)	3	4	6	8	9
EtOH (% m/m) (X_3)	33	40	50	60	67

T (°C) (X1) = Temperature; SLR (mg/mL) (X2) = solid-liquid ratio (*H. oreadica* powder leaves/ EtOH); EtOH (% m/m) (X3) = Alcoholic strength

RESULTS

Volatiles content, total ashes, and insoluble acids ashes

Volatiles content level was 8.44 ± 0.06%, total ashes were 2.46 ± 0.0010%, and insoluble acids ashes were 0.06 ± 0.0007%.

Particle size distribution and intumescence index

The *H. oreadica* powder leaf presented granulometry between 150 µm to 1000 µm, with retention of 47.38% and 47.51% in the diameters of 425 µm (tamis 40) and 250 µm (tamis 60), respectively. The intumescence index obtained was 3 mL.

Phytochemical screening

In the leaf powder were verified coumarins, cyanogenetic heterosides, digitalis, flavonoids, saponins, and tannins (Table No. 1).

Table No. 3
The central composite rotatable design (CCRD) delineation of the TP and Fv ultrasound-assisted extractive process of *Hortia oreadica* leaves

Assays	T (ºC) X_1	SLR (mg/mL) X_2	EtOH (%) X_3	TP (mg/mL)	Fv (mg/mL)
1	40	4	40	0.079	0.049
2	40	4	60	0.099	0.069
3	50	4	40	0.092	0.054
4	50	4	60	0.101	0.053
5	40	8	40	0.149	0.076
6	40	8	60	0.173	0.088
7	50	8	40	0.151	0.068
8	50	8	60	0.177	0.068
9	45	6	33	0.128	0.044
10	45	6	67	0.125	0.058
11	37	6	50	0.134	0.062
12	53	6	50	0.134	0.063
13	45	3	50	0.067	0.035
14	45	9	50	0.173	0.080
15	45	6	50	0.127	0.063
16	45	6	50	0.119	0.057
17	45	6	50	0.124	0.063
18	45	6	50	0.123	0.058
19	45	6	50	0.127	0.065
20	45	6	50	0.135	0.065

T (°C) (X1) = Temperature; SLR (mg/mL) (X2) = Solid-liquid ratio (*H. oreadica* powder leaves/EtOH); EtOH (% m/m) (X3) = Alcoholic strength; TP (mg/mL) = Total phenols; Fv (mg/mL) = Flavonoids

Table No. 1
Summary of the phytochemical screening in *Hortia oreadica* leaves.

Metabolites	Presence
Alkaloids	-
Anthraquinones	-
Digitalis	+
Flavonoids	+
Saponins	+
Tannins	+
Coumarins	+
Cyanogenetic heterosidases	+

Quantitative analysis of total phenols (TP) and flavonoids (Fv) and extraction optimization

The total phenols ranged from 0.079 to 0.177 mg/mL and flavonoids ranged from 0.035 to 0.088 mg/mL (Table No. 3). By the desirability function approach, it was verified that the best-predicted extraction condition would be at 40ºC using 60% hydroalcoholic solution (w/w) and a solid-liquid ratio of 8 mg/mL ($p<0.05$).

From the multiple regression data analysis of total phenolic compounds, it was observed that the factors evaluated did not present significant

interaction at the 5% level. However, their respective linear effects and quadratic effect of the SLR variable were included in the model. The value of *p* for lack of fit was not significant, showing that the model presented adequate adjustment. The value of R2adj was 0.9438, indicating that the model showed a relevant predictive and explainability power that can be described by:

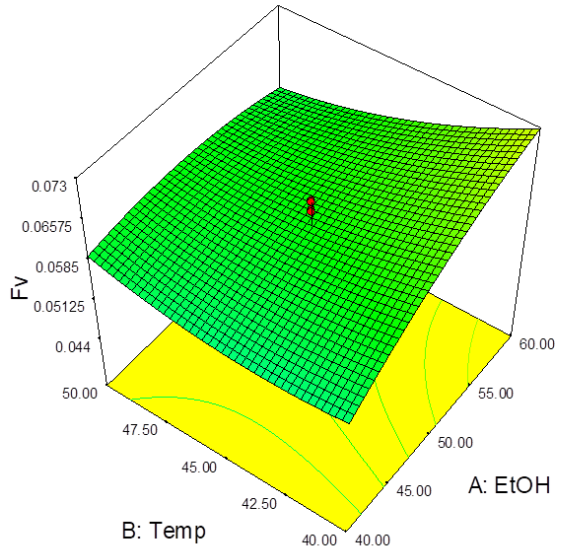
$$TP = 0.24165 + (5.43484 \times 10^{-4}) \times EtOH - 0.011226 \times Temp + 0.016798 \times SLR + (1.28526 \times 10^{-4}) \times Temp^2$$

For the Fv response variable, the variance analysis reveals that the interaction between the EtOH and temperature variables was significant at the 10% level (p = 0.0732), and the behavior of these two variables together can be visualized in the plot of response surface at Figure No. 1. From this plot, it is possible to visualize that lower temperatures associated with higher alcoholic strength have higher concentrations of total flavonoids. The R^2_{adj} value for this model was 0.7075. Therefore, the model for this behavior can be described by:

$$Fv = -0.037681 + (6.30333 \times 10^{-3}) \times EtOH - (3.94376 \times 10^{-3}) \times Temp + (5.51892 \times 10^{-3}) \times SLR - (8.47909 \times 10^{-5}) \times EtOH \times Temp - (2.09685 \times 10^{-5}) \times EtOH2 + (8.49714 \times 10^{-5}) \times Temp^2$$

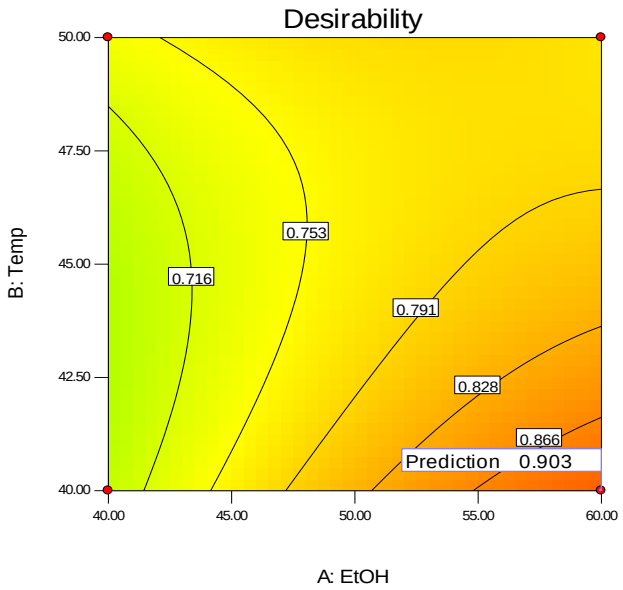
Using the desirability function approach, a D = 0.903 value was obtained, revealing that the geometric combination of the two variables with the best conditions suggested by the model was: alcoholic strength at 60%, the temperature at 40°C, and 8 mg/mL solid-liquid ratio. The contour plot (Figure No. 2) represents the variation of D values as a function of temperature and EtOH levels. From this plot, it is possible to notice that higher desirability values are found in higher alcoholic gradations and lower temperatures.

Figure No. 1



Response surface plot: flavonoids (Fv) as a function of the variables: temperature (Temp), alcoholic strength (EtOH)

Figure No. 2



Contour plot representing the overall desirability of the following responses: total phenols (TF) and flavonoids (Fv) as a function of the variables: temperature (Temp) and alcohol (EtOH)

Global desirable function (D) verification

The values predicted by the global desirability function were verified in triplicate for the combination of factors alcoholic strength at 60%, temperature at 40°C, and SLR of 8 mg/mL. The following experimental concentrations were obtained: 0.171 ± 0.002 mg/mL for TF (predicted value = 0.165) and 0.087 ± 0.002 mg/mL for Fv (predicted value = 0.084). For TF, the 5% difference between the experimental and predicted results for this assay was observed. For the variable Fv, the difference of 4.6% between predicted and experimental values was observed. Thus, the agreement between the experimental and predicted results obtained by the desirability function was verified.

DISCUSSION

Despite its therapeutic and technological potential, the plant species *H. oreadica* is poorly studied. Its quality parameters had not been established yet. Excessive moisture in plant raw materials allows enzymes that can degrade chemical constituents, besides allowing the development of fungi and bacteria. The maximum moisture content established in different pharmacopeias varies between 8 and 14%. Hence, the moisture content of *H. oreadica* (volatile content = 8.44 ± 0.06%) is within the parameters described for other species (Soares & Farias, 2017). Furthermore, the total ash content determination enables the presence of non-volatile inorganic impurities, which may be present as contaminants, to be verified. And the content of ash insoluble in hydrochloric acid allows, for example, to verify soil or sand residues (Soares & Farias, 2017). The Brazilian Pharmacopoeia (Brasil, 2010b) recommends, in the monographs of vegetal drugs constituted by leaf, total ash contents varying between 30% to 4%; and acid-insoluble ash contents ranging from 12.0% to 0.4%. In this sense, *H. oreadica* is within the parameters described for other species (total ash contents = 2.46 ± 0.0010%; acid-insoluble ash = 0.06 ± 0.0007%).

According to the Brazilian Pharmacopoeia (Brasil, 2010a), the particle size range from *H. oreadica* leaf was classified as a semi-thin powder (the one whose particles pass entirely through the nominal opening aperture mesh of 355 mm and, at most, 40% by the sieve with a little aperture of 180 mm mesh). The powder particles' size determines the contact surface for the interaction with the extracting liquid, making it possible to choose the appropriate extraction process for this raw material. Its determination is an essential step in the extractive processes to ensure the extraction yield and the quality of the extract (Migliato *et al.*, 2007; Hubinger *et al.*, 2009; Alves *et al.*, 2010).

The swelling index determination is a method to indicate the number of polysaccharides present in the raw material (Soares & Farias, 2017), being essential to predict the volume of solvent that must be added during the production of these extracts (Couto *et al.*, 2009). The swelling index for the powder of *H. oreadica* leaves was 3 ml, suggesting the presence of mucilage and gum (Frasson *et al.*, 2003; Barroso & Oliveira, 2009).

It was verified the presence of coumarins, cyanogenetic heterosidases, digitalis, flavonoids, saponins, and tannins in the *H. oreadica* leaf. Many phenolic compounds have been identified in *H. oreadica* leaf (Severino *et al.*, 2009; Braga *et al.*, 2012). Although the presence of alkaloids in species of Rutaceae is expected, this metabolite was not found in the present study. In previous studies with *H. oreadica* leaf, alkaloids were reported (Severino *et al.*, 2009; Braga *et al.*, 2012; Severino *et al.*, 2014). This divergence may be due to differences in soil composition, seasonality, or individual variations (Gobbo-Neto & Lopes, 2007) since the samples were collected in different regions and at different times of the year.

Identifying and quantifying metabolite classes is an essential strategy for quality control of raw materials and products of natural origin (Soares & Farias, 2017). The ultrasound-extractive method is an alternative approach to extract phenols and flavonoids with the advantage of shorter extraction time and optimization of solvents and raw material amounts, reducing degradation risks of the components by oxidation, thermal stress, or photodegradation (Costa, 2001b; Azwanida, 2015). However, optimizing this extraction without a statistical tool is a time-, solvent-, and material-consuming task. The optimization of phenol and flavonoids extraction using CCRD is considered the most convenient design in RSM because it allows fast evaluation of the conditions ranges and indicates the role of each parameter (Chen *et al.*, 2015; Izadiyan & Hemmateenejad, 2016; Hayder *et al.*, 2021; Yusof *et al.*, 2021; Zakaria *et al.*, 2021). This statistical approach demonstrated to be efficient also for Cerrado plant species.

CONCLUSION

The present work provided the physicochemical characterization of the *H. oeradica* leaf powder and the physicochemical description of the *H. oeradica* leaf hydroethanolic extract (obtained by percolation), standard data that can be used as parameters of raw material quality. In leaf powder, coumarins, cyanogenetic heterosidases, digitalis, flavonoids, saponins, and tannins were identified. The ultrasound-assisted extraction of phenolic and flavonoid compounds was optimized by the CCRD, employing a desirability function approach. It was found ideal for extracting phenols and flavonoids from *H. oeradica* leaf: alcoholic strength at 60%, the temperature at 40°C, and 8 mg/mL of SLR. Due to the popular use and the potential therapeutic use of *H. oreadica*, the optimized extraction parameters obtained in this study could be upscaled to develop pharmaceutical drugs to treat bacterial and parasitic infections, hepatopathies, and gastropathy, among others. In addition, further bioprospecting studies of new bioactive molecules and biological activities of H. oeradica are urgent.

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