Ciência e Agrotecnologia
SCIENCE AND AGROTECHNOLOGY
Ciência e Agrotecnologia, 44:e017420, 2020
http://dx.doi.org/10.1590/1413-7054202044017420
Agricultural Sciences
eISSN 1981-1829

Maceration extraction conditions for determining the phenolic compounds and the antioxidant activity of *Catharanthus roseus* (L.) G. Don

Condições de extração por maceração para determinação de compostos fenólicos e atividade antioxidante de *Catharanthus roseus* (L.) G. Don

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Received in June 16, 2020 and approved in October 8, 2020

ABSTRACT

Catharanthus roseus (L.) G. Don is a medicinal plant known for its important constituent aromatic compounds. In the literature, no detailed studies elucidating the methods of extraction of phenolic compounds for this species are available. Therefore, the present study was aimed at evaluating the maceration parameters for determining the total phenols content in *C. roseus*. The parameters of plant mass, extraction phase composition, homogenization type, extraction time, and temperature were evaluated in terms of improving the extraction conditions. The plant extracts were subjected to specific spectrophotometric analysis designed for each parameter, and the total phenols content was determined. After establishing the optimal conditions, the extracts were prepared from *C. roseus* cultivars, followed by evaluation of antioxidant activity using spectrophotometry, phenolic acid content using GC–MS, and dissimilarity using Unweighted Pair Group Method in combination with the Arithmetic Mean method. The optimal extraction conditions obtained were: 30 mg of dry plant tissue in 0.72 mL of extraction phase ethanol:water, 50:50 (v/v) at 50 °C for 1 h. The average total phenols content was 30.3 mg g^{-1} of gallic acid equivalents (GAE) and 20.0 mg g^{-1} of flavonoid quercetin equivalents (QE). The antioxidant activities determined using the DPPH, ABTS, and FRAP methods were 19.4, 11.8, and 23.3 mg g^{-1} of Trolox equivalent antioxidant capacity (TEAC), respectively. There was a predominance of ferulic (42%–56%) and caffeic (21%–52%) acids in the leaf extracts of *C. roseus*. The extraction process increased the total phenol content by 55%, from 18 to 28 mg GAE.

Index terms: Phytochemistry; DPPH; ABTS; FRAP; phenolic acids.

RESUMO

Catharanthus roseus (L.) G. Don é uma planta medicinal conhecida por importantes compostos aromáticos constituintes. Na literatura, não há estudos detalhados que elucidem os métodos de extração de compostos fenólicos para esta espécie. Portanto, o presente estudo teve como objetivo avaliar os parâmetros de maceração para determinar a concentração de fenóis totais em *C. roseus*. Os parâmetros de massa de planta, composição da fase extratora, tipo de homogeneização, tempo de extração e temperatura foram avaliados para melhorar as condições de extração. Os extratos das plantas foram submetidos à análise espectrofotométrica para cada parâmetro e a concentração de fenóis totais foi determinada. Após estabelecer condições ótimas, os extratos foram preparados a partir de cultivares de *C. roseus*, seguidos da avaliação da atividade antioxidante por espectrofotometria, conteúdo de ácidos fenólicos por CG-EM e dissimilaridade pelo método de pares de médias aritméticas não ponderadas. As condições ótimas de extração obtidas foram: 30 mg de tecido vegetal seco em 0.72 mL de fase de extração etanol: água, 50:50 (v/v), a 50 ºC por 1 h. A concentração média de fenóis totais foi de 30.3 mg g^{-1} equivalentes de ácido gálico (EAG) e 20.0 mg g^{-1} equivalentes de quercetina (EQ) para flavonoides. A atividade antioxidante determinada pelos métodos DPPH, ABTS e FRAP foi de 19.4, 11.8 e 23.3 mg g^{-1} de concentração equivalente de Trolox e atividade antioxidante (CETAA), respectivamente. Houve predomínio de ácidos ferúlico (42%-56%) e cafeico (21%- 52%) nos extratos de folhas de *C. roseus*. O processo de extração aumentou o teor de fenóis totais em 55%, de 18 para 28 mg EAG.

Termos para indexação: Fitoquímica; DPPH; ABTS; FRAP; ácidos fenólicos.

INTRODUCTION

Catharanthus roseus (L.) G. Don is a plant species belonging to family Apocynaceae (Moon et al., 2018), and is popularly known as vinca, maria-sem-vergonha, or boa-noite in Brazil. This species has provided two important alkaloids with antineoplastic function– vincristine and vinblastine (Falcão et al., 2017; Leonti et al., 2017), and

is, therefore, recognized for its medicinal properties in various parts of the world, including India, China, and the European and American nations (Kaushik et al., 2017). Although there are several studies on the use of *C. roseus* for cell cultures, indolic alkaloids, and nanoparticles (Abouzeid et al., 2019; Osibe; Aoyagi, 2019; Azhar et al., 2020), to the best of our knowledge, no detailed studies elucidating the methods of extraction of phenolic compounds from this species are reported so far.

The phenolic compounds present in plants provide protection against different environmental stresses, such as excessive solar radiation and attack by pathogens and pests (Speed et al., 2015). In addition, these compounds may serve as potent antioxidant agents which could assist in combating aging and health problems occurring in humans (Ćujić et al., 2016).

Among these phenolic compounds, salicylic, ferulic, gallic, and chlorogenic acids, also known as phenolic acids, are of particular importance as they contribute greatly to the antioxidant activity in plants (Suriano et al., 2018). Moreover, these phenolic acids may be utilized as chemical markers for differentiating species or cultivars (Chen et al., 2016). The extraction of these compounds from plants is a crucial step; even then, no consensus has been reached so far on a standard extraction method (Ćujić et al., 2016; Wianowska; Gil, 2019).

One of the various extraction methods is microwave extraction, which uses dipolar rotation and ion conduction for sample heating to promote the migration of the target compounds to the extraction phase (Vinatoru et al., 2017). Another method is the ultrasound extraction, which is based on the use of low frequency waves to cause cavitation with high energy bubbles combined with pressure and temperature (Freitas et al., 2015; Vinatoru et al., 2017). Pressurized liquid extraction involves applying high pressure and temperature to maintain the solvent in a liquid state and increase the penetration into the matrix, ultimately favoring the extraction of the compounds of interest (Castro-Puyana et al., 2013). Extraction with supercritical fluid is based on the use of substances that are maintained above their critical point using pressure and temperature; carbon dioxide is the most widely used supercritical fluid for the extraction of organic compounds (Da Silva et al., 2016).

Although the above-stated methods have provided efficient extractions, they are either highly expensive or/and have certain disadvantages. For instance, the energy applied in microwave-assisted extraction may cause oxidation of the lipids present in the matrix, thereby interfering with the final product (Pharm; Pharm; Pharm, 2015). Ultrasound extraction may also involve deleterious effects on the target compounds according to the applied energy and extraction time. Other techniques involving complex operations with several parameters require extensive investigation prior to application (Arsad et al., 2014).

Traditional methods, such as maceration, stirring-assisted maceration, and Soxhlet extraction, are the most used ones throughout the world (Alara et al., 2018; Belwal et al., 2018; Ćujić et al., 2016). Maceration extraction is based on solid–liquid separation, with an organic solvent or water as the liquid phase. The main solvents used for the extraction of phenolic compounds are methanol, ethanol, water, or a mixture of these solvents (Ćujić et al., 2016; Vajić et al., 2015). It is worth stating here that, to date, no consensus has been reached on a standard solvent for the extraction of phenolic compounds from plants. The efficiency of extraction using maceration could be enhanced by analyzing homogenization, time, and temperatures (Ghomari et al., 2019). However, maceration extraction also has certain limitations, such as a long extraction period and the requirement of large plant masses and consequently, large amounts of solvents (Alara et al., 2018). These parameters were, therefore, evaluated in the present study.

The objective of the present study was to evaluate the maceration parameters for determining the total phenols content in *C. roseus*. The sample extracts were prepared under optimal conditions from 12 *C. roseus* cultivars, and were subjected to evaluation of antioxidant activity using spectrophotometry, phenolic acid content using GC–MS, and dissimilarity using Unweighted Pair Group Method in combination with Arithmetic Mean (UPGMA) method.

MATERIAL AND METHODS

Reagents and Instrumentation

Methanol, ethanol, and acetone PA grade obtained from Vetec (Rio de Janeiro, Brazil) were used as solvents for extraction. The total phenolics content was measured using 99% pure gallic acid as a standard (Sigma-Aldrich, St. Louis, USA), while the quercetin standard obtained from Sigma-Aldrich (St. Louis, USA) was utilized to determine the flavonoid concentration. The reagents used for determining the antioxidant activity were: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), and Trolox, all with a purity of 99%. All the reagents were obtained from Sigma-Aldrich (St. Louis, USA)**.** Vanillic (purity: ≥97%), syringic (≥98%), ferulic (≥99%), and

quercetin (≥95%) acids were also purchased from Sigma-Aldrich (St. Louis USA). Salicylic acid (99.8%) was obtained from Vetec (Rio de Janeiro, Brazil).

The following equipment was employed in extract preparation: (1) a Phoenix vortex (São Paulo, Brazil); (2) water purifier from Millipore (São Paulo, Brazil); (3) water bath with a provision for agitation and heating, from Nova Ética (São Paulo, Brazil); and (4) a heating plate from Nova Ética (São Paulo, Brazil). The total phenols and flavonoid contents and the antioxidant activity were determined using an absorption spectrophotometer operated in UV–Vis region (Cary 60, Agilent Technologies, Australia).

Sample collection and preparation

The optimal extraction conditions were determined using *C. roseus* leaves collected at latitude 16º51'38'' S, longitude 44º55'00'' W, and an altitude of 652 m. The collected leaves were washed with ultrapure water and oven-dried at 60 °C for 20 h, following which they were ground to a fine powder, sieved through a 500-µm sieve, and stored until the extraction.

Extraction conditions

The initial extraction of phenolic compounds was based on the method described by Ma et al. (2011), with certain modifications. Briefly, 1 g of dried *C. roseus* leaves was macerated with 8 mL of extracting phase comprising ethanol and acetone (ethanol:acetone, 70:30, v/v). The mixture was homogenized in a bath at 37 °C under stirring conditions at 150 rpm for 1 h. The homogenized mixture was filtered, and the residue left behind was subjected to two identical extractions. Subsequently, the filtrates obtained from the three extractions were combined, diluted five times, and stored at −20 °C protected from light until used for UV–Vis spectrophotometric analysis.

With the objective of improving the extraction conditions for obtaining phenolic compounds from *C. roseus*, the evaluation of plant mass, extraction phase composition, homogenization type, extraction time, and the temperature was performed. The sample mass was the first parameter evaluated, for which the initial extraction was repeated with a sample:solvent ratio of 1:24 and by varying the mass and volume. The same extraction process was performed for evaluating the second parameter, with the same mass as selected for the previous parameter, followed by evaluating the different compositions of the extractor phase. Homogenization, with agitation (vortex and mechanical homogenization bath at 150 rpm) or without agitation (system left to stand undisturbed for 24 h), was performed using the best extraction phase determined in the previous step, in order to obtain the third parameter. Next, the extraction time was evaluated, which was followed by the evaluation of the extraction temperature. The evaluated levels of each of these parameters are listed in Table 1.

In the case of each parameter evaluation, the extract obtained after the extraction process was subjected to spectrophotometric analysis to determine the total phenols content, which was then subjected to analysis of variance using F-test at 5% significance level in R-studio software program (version 1.0.153). Significant means were compared using Duncan's test (5%). At each stage, the extractions were performed in triplicate, and each repetition involved four absorbance readings for total phenols content.

Table 1: Parameters with their evaluated levels for the extraction of phenolic compounds from *C. roseus*.

Parameters	Levels
Plant mass	30, 60, 120, 250, 500 and 1000 mg
Extraction phase composition	Acetone Ethanol Methanol Water Ethanol:acetone (70:30. v/v) Ethanol:water (50:50. v/v) Methanol:water (50:50. v/v)
Homogenization	Vortex for 5 min Maceration for 24 h (without stirring) Maceration for 3 h (with stirring)
Extraction time	1, 3, 12, 24 and 48 h
Temperature	30, 40, 50 and 60 ºC

In the final maceration process, the extracts were prepared in triplicate from 12 *C. roseus* cultivars: Vinca Cora Red, Vinca Sustorm White Eye, Vinca Sustorm Pure White, Vinca Sustorm Blush, Vinca Sustorm Rose Eye, Vinca Cora Strawberry, Vinca Sustorm Deep Orchid, Vinca Cora Deep Lavender, Vinca Sustorm Purple, Vinca Sustorm Apricot, Domestic Hybrid, and wild Hybrid. The seeds of Domestic Hybrid and Wild Hybrid were obtained from a domestic garden in the northern region of Minas Gerais, while the rest of the cultivars were purchased from a commercial company in the state of São Paulo. All the cultivars were planted under identical conditions.

The extracts obtained from these cultivars were subjected to spectrophotometric analysis to determine the total phenols and flavonoid concentrations and the antioxidant activity. The results were presented as means and standard errors and were analyzed using Duncan's test (5%). In addition, the chemical compositions of the constituent phenolic acids in these extracts were evaluated using gas chromatography coupled with mass spectrometry (GC–MS).

Evaluation of the total phenols and flavonoid concentrations and the antioxidant activity

The total phenols content was determined using the methodology proposed by Kim et al. (2003), with modifications. Briefly, 40 µL plant extract or gallic acid solution, 360 µL ultrapure water, and 40 µL Folin-Ciocalteu reagent were mixed, and the mixture was homogenized and then stored in darkness for 5 min. Subsequently, 400 µL of 7% (w/v) sodium carbonate solution and 160 µL ultrapure water were added to the mixture, forming a total volume of 1.0 mL. This mixture was homogenized and left undisturbed for 90 min at room temperature to allow for the reaction to complete. Quantification was performed on a UV–Vis spectrophotometer at 750 nm, and gallic acid was used as the standard in the concentration range of 1.0 to 8.0 mg L^{-1}. The results were expressed as equivalent mg of gallic acid/g dry plant sample.

Flavonoid concentration was determined using the method described by Boroski et al. (2015), with modifications. Briefly, 100 µL of vegetable extract or quercetin solution, 50 µL of 5% (w/v) aluminum chloride solution, and 850 µL of methanol were mixed in a microtube. The mixture with a reaction volume of 1.0 mL was homogenized and allowed to stand for 30 min. Quercetin was used as the standard to obtain the analytical curve in the concentration range of 1.0-12 mg L^{-1}. Quantification was performed on a spectrophotometer at 425 nm. The results were expressed as equivalent mg of quercetin/g dry plant sample.

The antioxidant activity was determined through DPPH method by following the methodology described by Brand-Williams, Cuvelier and Berset (1995), with modifications. First, 4.0 mg 2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved completely in 25 mL of methanol. The obtained solution was diluted with 80% (v/v) methanol to obtain a working solution with an absorbance of 1.10 ±0.02 measured at 515 nm. This solution was used to determine the radical scavenging activity of *C. roseus* extracts. Briefly, an aliquot of DPPH solution (830 µL) was transferred to a microtube containing 170 µL of plant extract or Trolox solution, and the mixture with a total volume of 1.0 mL was allowed to stand for 45 min. Trolox in methanol was used as the standard to obtain the analytical curve in the concentration range of 1.0-7.0 mg L^{-1}. The results were expressed as equivalent mg of Trolox/g dry plant sample.

The ABTS assay was performed according to the method described by Siddiq, Sogi and Dolan (2013), with modifications. The stock solution containing $ABTS^{+}$ cation radicals was obtained by mixing potassium persulfate solution (2.4 mmol L^{-1}) and ABTS solution (7.0 mmol L^{-1}) in a ratio of 1:1 (v/v); both solutions prepared in ultrapure water. The obtained solution was homogenized and left undisturbed for 16 h in the dark to allow for the formation of the radical. Afterward, the absorbance of the stock solution was measured at 734 nm, following which it was diluted with 80% (v/v) methanol to obtain a working solution with an absorbance of 0.70 ±0.02 at 734 nm. A volume of 990 µL of the working solution was transferred to a microtube containing 10 µL of plant extract or Trolox solution. A Trolox standard prepared in methanol was used to obtain the analytical curve in the range of 0.25–5.0 mg L^{-1}. The results were expressed as equivalent mg of Trolox/g dry plant sample.

The ferric ion-reducing antioxidant power (FRAP) of the plant extracts was determined using the methodology described by Boroski et al. (2015), with modifications. Three TPTZ stock solutions of 10 mmol L^{-1} in each of 40 mmol L^{-1} hydrochloric acid, 300 mmol L^{-1} sodium acetate buffer, and 20 mmol L^{-1} ferric chloride in water were prepared, and the working solution was obtained by mixing these three stock solutions in a ratio of 1:10:1 (v/v/v). The obtained solution was homogenized and allowed to stand in a heating bath at 37 °C without stirring for 3 h. The reaction was performed in a microtube containing 30 µL of the extract or the standard, 90 µL ultrapure water, and 900 µL TPTZ working solution. The mixture with a total volume of 1.02 mL was homogenized and allowed to stand for 45 min at 37 °C. Analyses were performed

using a spectrophotometer at 593 nm. Trolox in methanol was used as the standard to obtain the analytical curve in the concentration range of 1.0-7.0 mg L^{-1}. The results were expressed as equivalent mg of Trolox/g dry sample.

Determination of the chemical composition of phenolic acids

In order to determine the chemical composition of the phenolic acids obtained from plant extracts, it was necessary to first perform a derivatization step prior to the main step of gas chromatography coupled to mass spectrometry (GC–MS) analysis.

The derivatization of the extracts involved mixing 1.0 mg of the extract, 100 µL of BSTFA, and 60 µL of anhydrous pyridine in a conical flask, followed by shaking and heating to 50 °C for 30 min. Subsequently, the reaction mixture was transferred to an injection vial with an insert and subjected to GC–MS analysis.

Chromatographic analysis was performed on an Agilent Technologies gas chromatography system (GC 7890A) coupled to an Agilent Technologies (Australia) mass detector (MS5975C), using Helium (99.9999% purity) as carrier gas at a rate of 1.0 mL min^{-1}. The sample (1.0 µL) was injected into the chromatography column using an auto-injector (CTC combiPaL) in splitless mode. The injector was maintained at 290 °C. The capillary column used was DB-5MS (30 m × 0.25 mm × 0.25 µm; Agilent technologies). Initially, the GC oven was at 100 °C, after which it was heated to 150 °C at the rate of 10 °C min^{-1}. After 150 °C, the temperature was raised at a rate of 5 °C min^{-1} to 225 °C, which was followed by heating to 300 °C with an increase of 20 °C min^{-1}. The oven temperature was maintained at 300 °C for 2 min. The interface temperature was maintained at 280 °C and the electron impact ionization at 70 eV. The phenolic compounds in the extracts were identified by comparing the standard mass spectra with the apparatus library (NIST 2.0). The subsequent analyses followed the selective mode of ions. The selected ions were: *m/z* 135, 267, 268 (salicylic acid); *m/z* 267, 297, 312 (vanillic acid); *m/z* 312, 327, 342 (syringic acid); *m/z* 281, 443, 458 (gallic acid); *m/z* 338, 381, 396 (ferulic acid); and *m/z* 219, 381, 396 (caffeic acid).

The results of the quantification of phenolic acids in the extracts using GC–MS were expressed as mean and standard deviation. The mean values were then used to study dissimilarity among the *C. roseus* cultivars using Unweighted Pair Group Method in combination with Arithmetic Mean (UPGMA) method in R-Studio software program.

RESULTS AND DISCUSSION

Extraction conditions

Maceration extractions are preferred as they provide the advantage of handling large solvent volumes and sample mass (Alara et al., 2018). However, extractions requiring large volumes and mass have emerged as the main barrier in case of certain plants, such as *C. roseus*, in the cultivation systems in vases, *in vitro*, or under salt stress, which provide a total dry matter of less than 2.1 g $plant^{-1}$ (Chung et al., 2007; Jaleel et al., 2008). Therefore, the first step in the process of determining the optimal conditions for the extraction of phenolic compounds from this plant was to determine the most suitable plant sample mass. The total phenols concentrations obtained for each evaluated mass value are presented in Figure 1.

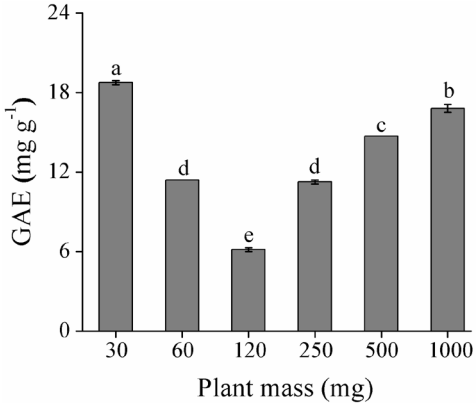

Figure 1: Total phenols concentrations (mean ± standard error; *n = 3*) obtained for six different leaf mass values for *C. roseus*. The concentration values are expressed as gallic acid equivalents (GAE). The conditions represented by the same letter do not differ statistically according to Duncan's test at 5% significance level.

It was observed that the extract from 30 mg of *C. roseus* presented the highest total phenols content, which differed statistically from the others. The interaction between sample particles and the extraction phase observed for this mass (30 mg) was greater than that observed for a mass of 1000 mg. Sample agglomeration in larger masses reduces interaction with the solvent, and consequently, the extraction efficiency (Luthria, 2012). The total phenols content obtained for the mass of 30 mg *C. roseus* in the present study was higher than the value obtained for

Guayule leaf phenols (15 mg GAE g^{-1} sample) in a study (Piana et al., 2018) that used a sample mass 80 times larger than that used in the present study. Therefore, the mass of 30 mg was selected for further analyses in the present study.

The second step involved evaluating the most commonly used solvents reported in the literature to determine the best extraction phase composition. Seven extraction phases were evaluated, and the total phenols concentrations obtained for each of them are presented in Figure 2.

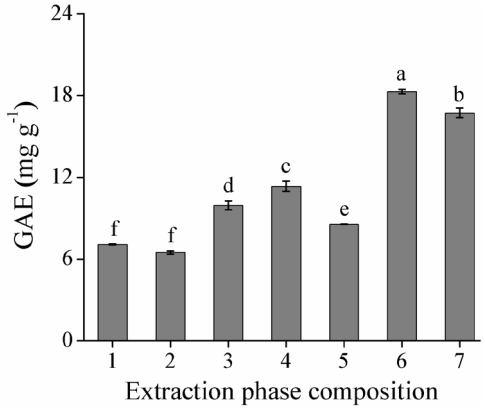

Figure 2: Total phenols concentrations (mean ± standard error; *n = 3*) obtained for the seven extraction phases: 1: acetone, 2: ethanol, 3: methanol, 4: water, 5: ethanol:acetone (70:30, v/v), 6: ethanol:water (50:50, v/v), and 7: methanol:water (50:50, v/v). The values are expressed in terms of gallic acid equivalents (GAE). The conditions represented by the same letter do not differ statistically according to Duncan's test at 5% significance level.

It was observed that the highest total phenols concentration in the extract was obtained with the ethanol:water (50:50, v/v) extraction phase. This extraction phase demonstrated a statistical difference in relation to the others in terms of concentration. The result was, on an average, 62% higher than that obtained using a single solvent. A similar result was obtained in a study concerning the phenols in chokeberries, in which the maximum phenols concentration was obtained using 50% ethanol (Ćujić et al., 2016). The combination of an organic solvent with water promotes sample hydration, loosens the cell walls, and facilitates the migration of phenolic compounds from the plant material to the extraction phase (Alara et al., 2018). Therefore, the extraction phase comprising ethanol:water (50:50, v/v) was selected for further steps.

The homogenization effect was the third parameter evaluated, and the results are presented in Figure 3.

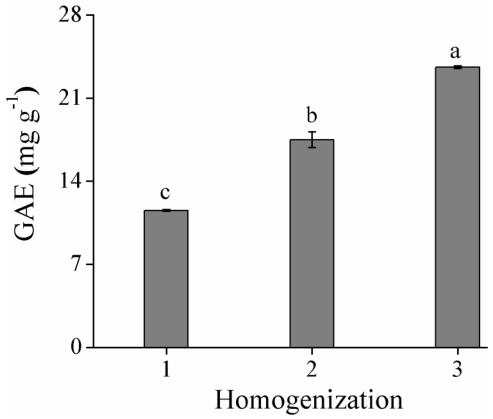

Figure 3: Total phenols concentrations (mean ± standard error; *n = 3*) obtained upon maceration with and without homogenization. 1: vortex (5 min), 2: bath with stirring (3 h), and 3: maceration without stirring (24 h). The values are expressed in terms of gallic acid equivalents (GAE). The conditions represented by the same letter do not differ statistically according to Duncan's test at 5% significance level.

Maceration without stirring produced extracts with higher total phenols concentration and differed significantly from the other modes of homogenization in the significance test. The present study is the first to report that agitating the system did not increase the efficiency of the maceration process. According to a previous study, the extraction of phenolic contaminants in water using the electromedical microextraction method was more efficient when conducted without agitation. It was indicated that agitation at a slow speed (~ 200 rpm) or rapid speed (~ 800 rpm) negatively affected the migration of the sample compounds to the extraction phase due to unknown reasons (Chong et al., 2018).

The total phenols concentration obtained with maceration at rest in the present study was higher than that obtained in a recent study on *C. roseus* using ultrasonic extraction (18 mg GAE g^{-1} of dry sample) (Pham et al., 2018). Therefore, maceration without stirring was selected for further steps.

Traditionally, the maceration extraction period ranges from 1 h to 5 d (Alara et al., 2018; Ćujić et al., 2016). However, prolonged extraction periods may result in the degradation of certain compounds present in the extract (Mojzer et al., 2016; Mokrani; Madani, 2016). Therefore, the extraction period was also evaluated in the present study, and the results are presented in Figure 4.

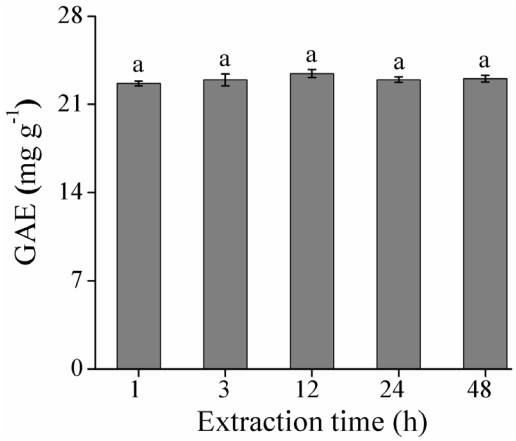

Figure 4: Total phenols concentrations (mean ± standard error; *n = 3*) obtained for five different extraction times. The values are expressed in terms of gallic acid equivalents (GAE). The conditions represented by the same letter do not differ statistically according to Duncan's test at 5% significance level.

As shown in Figure 4, there was no significant difference among the phenols concentration values obtained for the extracts at each extraction time. This result is consistent with the second law of Fick's Diffusion, according to which the migration of analytes ceases to occur after a certain period of contact, and a state of equilibrium is attained between the matrix and the extraction phase (Mokrani; Madani, 2016). According to the results, 1 h was selected as the extraction time for further steps. The same extraction period/time was reported for the extraction of total phenols from chokeberry (Ćujić et al., 2016) and lycopene extraction from industrial tomato peel residue (Kehili et al., 2019).

Next, the optimal temperature for the extraction process was evaluated, and the results are presented in Figure 5.

The extraction of phenolic compounds from *C. roseus* at the temperatures of 50 °C and 60 °C did not differ statistically and presented the highest total phenols concentration when compared with extraction at room temperature (30 °C). These findings indicated the importance of heat during extract preparation. Heating during the extraction process reduces the viscosity and surface tension of the solvents, promoting greater interaction between the extracting phase and the sample particles, thereby increasing the mass transfer rate of the analytes to the extract (Dai; Mumper, 2010). In addition, heating promotes the release of the phenolic acids linked to glycosides, thereby increasing the total extractable phenol content (Mokrani; Madani, 2016). The total phenols content values obtained in the present study (21.0–25.0 mg g^{-1}) were higher than those reported for the extraction of phenolic compounds from *Vinca rosea* leaves (15.9 mg g^{-1}) by performing maceration at 90 °C (Proestos et al., 2005), which indicates that the temperatures above 60 °C are capable of degrading the compounds of interest. Therefore, 50 °C was selected as the extraction temperature for further steps.

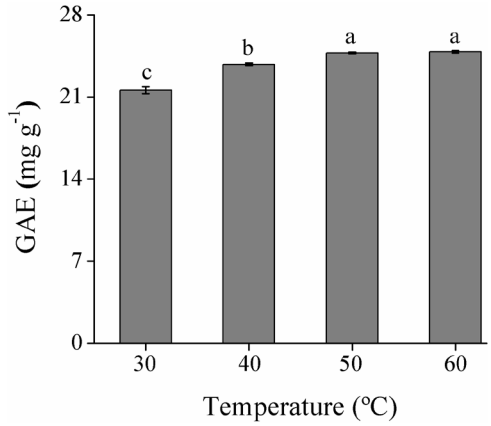

Figure 5: Total phenols concentrations (mean ± standard error; *n = 3*) obtained at four different extraction temperatures. The values are expressed in terms of gallic acid equivalents (GAE). The conditions represented by the same letter do not differ statistically according to Duncan's test at 5% significance level.

The results obtained in the analysis of the extracts from 12 *C. roseus* cultivars using the final optimal extraction conditions are listed in Table 2.

Analysis of variance performed using the 5% F-test was significant for analyzing total phenols, flavonoids, DPPH, ABTS, and FRAP in the *C. roseus* extracts. Cultivar 10 (Vinca Sustorm Apricot) was statistically different from the others in terms of its total phenols content. Cultivars 2, 3, 6, 8, 11, and 12 were not statistically different from each other and presented the lowest values of total phenols content. In the analysis of flavonoids concentration, Cultivar 1 and Cultivar 5 did not differ from each other, although presented significant differences relative to the other cultivars.

The mean total phenols content and the mean flavonoid concentration in *C. roseus* were 30.3 mg GAE g^{-1} and 20.0 mg QE g^{-1}, respectively. These results were superior to those reported for the aqueous extract of *C. roseus* leaves obtained from Patricia White cultivar, where the total phenols content of 18.1 mg GAE g^{-1} and flavonoids concentration of 10.0 mg QE g^{-1} were obtained using ultrasonic extraction (Pham et al., 2017). The results

of the present study may be explained by the presence of 50% ethanol in the extraction phase, as the use of binary mixtures favors the extraction compared to the use of a pure solvent (Daí; Mumper, 2010). In addition, the maceration method used in the present study allowed for the migration of phenolic compounds without the risk of degradation due to excess energy.

In regard to antioxidant activity, the highest Trolox equivalent concentrations were obtained with the FRAP method, followed by DPPH and ABTS methods, respectively. The values obtained in the present study were higher than those reported recently for *C. roseus* with ultrasonic extraction TEAC of 8.34 mg g^{-1} was observed for DPPH, 12.1 mg g^{-1} for ABTS, and 10.5 mg g^{-1} for FRAP (Pham et al., 2018).

The reaction mechanism in the FRAP method involves electron transfer and could, therefore, be influenced by the conditions of the reaction medium and the reaction time (Prior et al., 2005). The FRAP method is often applied to the extracts with hydrophilic molecules (Boroski et al., 2015), as it is favored by the extraction phase comprising ethanol:water (50%, v/v). Furthermore, the DPPH radical presents a steric impediment for the molecules that are considered large, as these molecules are prevented from interacting with the unpaired electron of nitrogen present in the radical structure (Ácsová et al., 2019).

Chemical composition of phenolic acids

A previous study evaluated the presence of phenolic compounds in plant extracts along with their antioxidant activities (Pham et al., 2017). In the present study, the extracts from each cultivar were analyzed using GC–MS, and the obtained chromatograms were quite similar. A representative chromogram of *C. roseus* extract from Cultivar 11, the Domestic Hybrid, is depicted in Figure 6.

The six phenolic acids detected in the extracts of 12 *C. roseus* cultivars and their quantification results are listed in Table 3.

Ferulic and caffeic acids were detected in higher concentrations in the extracts of 12 *C. roseus* cultivars, and the concentrations of the other acids were in the range of 0.44–4.3 μg g^{-1}. The extraction of phenolic compounds, including ferulic acid, is influenced by the extraction period, pH, and temperature (Kumar; Pruthi, 2014). The time and temperature parameters were evaluated in the present study as well, and could explain the superior results obtained in comparison to those reported for olive leaves (*Olea europaea L.*), with the ferulic, vanillic, and caffeic acid concentrations of 1.1 μg g^{-1}, 4.4 μg g^{-1}, and undetected, respectively (Guodong et al., 2019). The vanillic and syringic acid concentrations obtained in the present study were lower than those obtained for millet leaves (*Setaria italica*) using 1.0 g of sample for extraction (Xiang et al., 2019).

Table 2: Total phenols content (GAE: gallic acid equivalents), flavonoids concentration (QE: quercetin equivalents), and the antioxidant activity [expressed as Trolox equivalent antioxidant capacity (TEAC)] determined using DPPH, ABTS, and FRAP methods in the extracts of 12 *C. roseus* cultivars.

Cultivars	Total phenols	Flavonoids	DPPH	ABTS	FRAP
	GAE (mg g^{-1})	QE (mg g^{-1})	---------- TEAC (mg g^{-1}) ----------		
1	32.4±1b	22.4±1ab	17.8±0.1de	11.6±0.5c	16.4±0.1d
2	27.5±0.02d	20.7±0.5cd	16.9±0.1 g	11.7±0.4c	16.1±0.01e
3	27.8±0.5d	18.4±0.1f	17.3±0.2f	11.2±0.1c	28.1±0.01c
4	33.3±0.5b	16.7±0.2 g	22.5±0.02a	13.2±0.1a	16.3±0.01de
5	32.2±0.9b	22.7±0.2a	22.5±0.1a	11.5±0.7c	28.5±0.02b
6	27.7±0.2d	20.3±0.2cde	22.4±0.02a	11.1±0.1c	16.1±0.1de
7	33.7±0.4b	20.2±0.4cde	17.8±0.07d	11.1±0.1c	16.1±0.1e
8	28.0±0.01d	20.7±0.08cd	20.7±0.2c	12.7±0.1ab	28.7±0.02ab
9	30.1±1c	21.0±1bc	22.1±0.01b	12.0±0.09bc	28.8±0.01a
10	35.6±0.2a	19.2±0.4def	17.9±0.07d	11.7±0.1c	28.2±0.07c
11	28.2±1d	18.5±1f	17.5±0.2ef	11.8±0.08bc	28.7±0.3ab
12	27.2±0.9d	19.0±0.9ef	17.4±0.1f	12.0±0.05bc	16.3±0.01de

Values are expressed as mean ±standard deviation (*n = 3*). 1) Vinca Cora Red, 2) Vinca Sustorm White Eye, 3) Vinca Sustorm Pure White, 4) Vinca Sustorm Blush, 5) Vinca Sustorm Rose Eye, 6) Vinca Cora Strawberry, 7) Vinca Sustorm Deep Orchid, 8) Vinca Cora Deep Lavender, 9) Vinca Sustorm Purple, 10) Vinca Sustorm Apricot, 11) Domestic Hybrid, and 12) Wild Hybrid. Means having the same letter in the column did not differ statistically according to Duncan's test at 5% significance level.

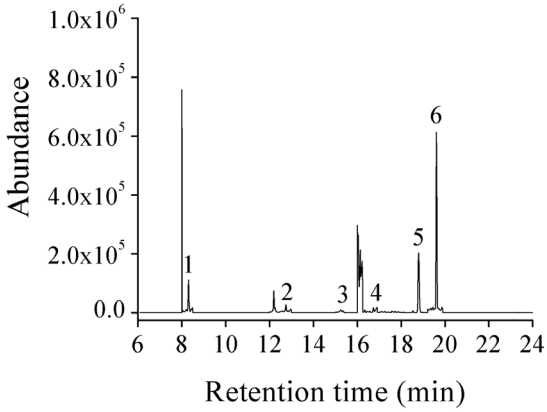

Figure 6: Chromatogram of Cultivar 11 (Domestic Hybrid) extract obtained under the final optimal extraction conditions: 1) salicylic acid; 2) vanillic acid; 3) syringic acid; 4) gallic acid; 5) ferulic acid; and 6) caffeic acid.

In addition to being directly associated with the antioxidant activity of the extracts, these phenolic compounds may serve as chemical markers for differentiating plant species or varieties (Assunção et al., 2019). In this context, the concentration values of these phenolic acids were subjected to cluster analysis of the *C. roseus* cultivars, and the dendrogram obtained using the UPGMA method is depicted in Figure 7.

The cut-off point established in the Mojena method was 19.755. In the cluster analysis, maximum dissimilarity was observed among the *C. roseus* cultivars, and two defined clusters were observed at this cut-off point: Cluster 1 included only Cultivar 11, while Cluster 2 included the rest of the cultivars.

The leaf extract of Cultivar 11 presented salicylic acid and ferulic acid concentrations that were four and two times higher, respectively, compared to the other cultivars. Salicylic acid is an important signaling molecule produced in response to biotic and abiotic stresses (Chavoushi et al., 2020), particularly in the defense response against pathogens and herbivory (Felton et al., 2018). Ferulic acid assists in response to pathogen infection and also contributes to the rigidity of cell wall by forming complexes in the lignification process (Kumar; Pruthi, 2014).

Both Cultivar 7 and Cultivar 11 correspond to "Vinca Sustorm Deep Orchid". However, the seeds of Cultivar 7 were acquired from a commercial company, while those of Cultivar 11 were obtained from a domestic garden. This difference in the seed origin could represent a gain from the adaptation perspective. Therefore, it was inferred that the different environmental conditions in which the seeds of these two cultivars were grown might have influenced developing a greater resistance to pathogens in Cultivar 11 compared to Cultivar 7.

Table 3: Phenolic acid concentrations in the extracts from 12 *C. roseus* cultivars.

Cultivars	Phenolic acids (μg g^{-1})					
	Salicylic	Vanillic	Syringic	Gallic	Ferulic	Caffeic
1	-	-	-	-	-	-
2	0.44±0.04	0.65±0.009	0.41±0.01	1.0±0.05	17±1	21±0.4
3	0.63±0.05	1.2±0.008	0.70±0.001	0.61±0.01	21±0.7	32±1
4	0.71±0.02	0.80±0.02	0.37±0.03	0.95±0.04	24±0.1	34±0.3
5	0.70±0.02	1.4±0.09	0.41±0.06	1.3±0.05	22±0.2	31±0.1
6	0.83 ±0.08	1.3±0.06	0.51±0.02	1.2±0.01	26±0.3	31±0.3
7	1.1±0.003	1.6±0.01	1.0±0.03	2.1±0.2	24±0.3	24±0.6
8	0.52±0.003	1.1±0.05	0.75±0.04	1.2±0.06	27±2	26±1
9	1.2±0.2	1.4±0.1	2.1±0.1	2.0±0.02	23±0.6	34±0.000
10	1.2±0.03	2.0±0.09	0.87±0.03	1.7±0.1	24±0.7	35±3
11	4.3±0.2	1.5±0.01	0.86±0.009	0.60±0.04	56±1	36±0.07
12	1.1±0.005	1.4±0.02	0.61±0.08	1.2±0.02	29±1	37±1

Values are expressed as mean ±standard deviation (*n = 3*). 1) Vinca Cora Red (not analyzed), 2) Vinca Sustorm White Eye, 3) Vinca Sustorm Pure White, 4) Vinca Sustorm Blush, 5) Vinca Sustorm Rose Eye, 6) Vinca Cora Strawberry, 7) Vinca Sustorm Deep Orchid, 8) Vinca Cora Deep Lavender, 9) Vinca Sustorm Purple, 10) Vinca Sustorm Apricot, 11) Domestic Hybrid, and 12) Wild Hybrid.

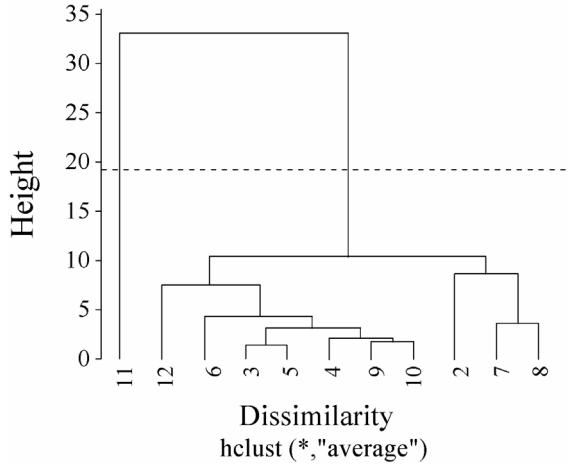

Figure 7: Cluster dendrogram obtained using the UPGMA method for the 12 *C. roseus* cultivars: 1) Vinca Cora Red (not evaluated), 2) Vinca Sustorm White eye, 3) Vinca Sustorm Pure White, 4) Vinca Sustorm Blush, 5) Vinca Sustorm Rose Eye, 6) Vinca Cora Strawberry, 7) Vinca Sustorm Deep Orchid, 8) Vinca Cora Deep Lavender, 9) Vinca Sustorm Purple, 10) Vinca Sustorm Apricot, 11) Domestic Hybrid, and 12) Wild Hybrid. The dashed line represents the Mojena cut-off point.

In Cluster 2, there were subdivisions defined by the differences in the concentrations of ferulic acid and caffeic acid. Cultivar 12 is abundant in nature and is, therefore, considered a wild cultivar. This cultivar was assigned to an isolated subdivision as it presented the highest concentrations of ferulic and caffeic acids. At the other end of the dendrogram, Cultivar 2 (Vinca Sustorm White Eye) was also placed in an isolated subdivision as it presented the lowest concentrations of these two acids.

CONCLUSIONS

The present study determined the optimal values of important parameters for the extraction of phenolic compounds from *C. roseus*. In addition, a significant reduction in the plant mass used as well as in the volume of extraction solvents required was accomplished without affecting the outcomes of the spectrophotometric and chromatographic analyses. The requirement of smaller sample mass and smaller solvent volumes is a positive step in regard to scientific and environmental scope. The study provides an efficient and safe alternative for performing extraction from the other plants or from structures other than leaves, such as flowers, roots, and seeds, which provide smaller amounts of plant material for the extraction process. The optimal extraction conditions determined in the present study are feasible for a conventionally-structured laboratory set-up, without requiring sophisticated equipment for sample preparation. In addition, the conditions may be applied for further detailed investigations concerning seasonality, environmental stress, and chemical markers for different cultivars. The present study would provide a foundation for future research on the extraction of phenolic compounds from *C. roseus* using other methodologies, such as response surface methodology.

ACKNOWLEDGEMENTS

To the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) (project number 420637/2016–8 and 308480/2019–8) and to the *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* (*FAPEMIG*) (APQ-01429–16) for the financial support and to the *Universidade Federal de Minas Gerais* (*UFMG*) for the financial support and for providing the infrastructure to conduct the study.

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