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Influence of extraction process on antioxidant activity and rutin content in *Physalis peruviana* calyces extract

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ABSTRACT

Physalis peruviana is an exotic fruit highly valued for its bioactive compounds. This work evaluates the effect of some variables of the extraction process of *P. peruviana* calyces extract (considered waste material) on total phenolic content, rutin content, and in vitro antioxidant activity of the extracts obtained. First, the influence of certain factors of the extraction process on the above mentioned response variables were analyzed. The response variables were then evaluated using in vitro assays such as NO (nitric oxide) and DPPH (1,1-diphenyl-2-picrylhydrazyl) uptake for antioxidant activity; the Folin-Ciocalteu method for total phenolic content; and the external standard method by HPLC-DAD for quantification of rutin. The results showed that the optimal conditions needed to obtain the best results for the response variables evaluated are the use of ethanol 70% as extraction solvent, and a percolation time of 72 hours, these being the factors that positively influenced the results.

INTRODUCTION

In recent decades, due to the large amount of research on phytochemistry and pharmacognosy, natural products from plant sources have gained particular importance in the treatment of different kinds of diseases (Gilani and Rahman, 2005; Patwardhan, 2005; Petronilho *et al*., 2012; Newman and Cragg, 2016). Cape gooseberry (*Physalis peruviana* L., Solanaceae) is an abundant specie of the South American Andes. The vegetal is an herbaceous, semi-upright shrub that is known for its round, orange-colored fruit. This fruit is about two inches in diameter and its juicy pulp, which has a pleasant smell and taste, is enclosed in five-sepal calyx (Wills *et al*., 1984). This species has become of great importance in Colombia, the world´s largest exported (Garzón, 2012), which is usually consumed fresh or in beverage preparation. However, the calyces are considered waste material after harvesting of the fruit. In this sense, investigations with this organ can contribute to better use of this plant material, giving higher added value to the species and its cultivation. Additionally, many medicinal properties have been attributed to *P. peruviana* in folk medicine, such as antispasmodic, diuretic, sedative, analgesic, antiseptic, and antidiabetic properties (Puente *et al*., 2011), and it is also used to treat malaria, asthma, hepatitis, dermatitis and rheumatism (Wu *et al*., 2006). The calyces, in particular, are widely used in traditional medicine to treat cancer, infections, fever and inflammation (Puente *et al*., 2011). In recent years, phytochemical studies of *P. peruviana* have shown the presence of phenolic compounds in different organs of this plant, such as the fruits and calyces, which correlates with the high antioxidant capacity of the plant (Licodiedoff *et al*., 2013; Toro *et al*., 2014; Vilaplana *et al*., 2014; Bravo *et al*., 2015). Different extraction methods are widely used to obtain crude extracts from plant materials. However, there is no universal extraction protocol because each plant or metabolite requires specific extraction conditions for optimal recovery of compounds, especially those responsible for biological activities. Effects of extraction process are, therefore, essentials to maintain and even

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improve positive effects on health. The aim of this study was to determine the best extraction conditions for calyces of *P. peruviana*, in order to obtain the highest rutin and total phenol contents, and better antioxidant capacity.

MATERIALS AND METHODS

Plant material

The calyces of *Physalis peruviana L.* were collected in the region of Granada Cundinamarca (2450 masl), on March, 2013. The fresh calyces were dried in an oven drying at a temperature of 40°C until constant weight, and ground in a knife mill. One specimen was stored in the Herbarium of the National University of Colombia (COL 512200).

Materials

Quercetin-3-*O*-rutinoside (rutin), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteau phenol reagent, ascorbic acid and gallic acid were purchased from Sigma-Aldrich® (St. Louis, MO, USA); sodium carbonate (Na_2CO_3), and methanol HPLC were supplied by J.T Baker® (Trinidad, Tobago) andacetic acid was obtained from Scharlau® (Sentmenat, Spain).

Preparation of extracts

For the extraction process, percolation with ethanol (10:150, m/v) was selected as the extraction method. The variables considered were the concentration of the extraction solvent (96 and 70% ethanol), the percolation time (48 and 72 hours), and the presence-absence of light..The number of tests and variations in combination were based of the experimental design suggested by statistical software R (Table 1). After extraction of the plant material, the extracts were evaporated to dryness in reduced pressure at 35°C.

Table 1: Experimental design matrix for preparation of *P. peruviana* calyces extracts.

Experiment	Duration of extraction (hours)	Presence of light during extraction	Extraction solvent
1	72	No	EtOH 70%
2	48	Yes	EtOH 96%
3	48	No	EtOH 96%
4	48	Yes	EtOH 70%
5	72	Yes	EtOH 70%
6	72	No	EtOH 70%
7	72	No	EtOH 96%
8	48	No	EtOH 70%
9	72	No	EtOH 96%
10	72	Yes	EtOH 96%
11	48	No	EtOH 70%
12	48	Yes	EtOH 96%

Determination of Total Phenolic Content (TCP)

The TPC in the extracts was determined according to the methodology previously described by Kappel and co-workers (2008), with slight modifications. Briefly, a 100 µL aliquot of extracts was assayed with 100 µL of Folin-Ciocalteu 2N reagent and 200 µL of sodium carbonate (7.5%). The resulting solution was vortex-mixed and diluted with distilled water to obtain a final volume of 2 ml. After 2 hours, the absorption was measured at 726 nm. Gallic acid was used as standard and the total phenolic content was expressed as mg of gallic acid/g of extract (Kappel *et al.*, 2008).

HPLC analysis

A Shimadzu® system (Shimadzu, Tokyo, Japan), consisting of a LC-6AD binary pump, SPD-M20A diode array detector (DAD), SIL-20A HT auto-sampler, DGU- 20As in-line degasser, and software LCsolution® were used. The injections (10 µL) were carried out on a Phenomenex C-18 10 µm (150 x 3.9 mm) column conditioned in a Shimadzu® CTO-20A column oven equilibrated at 35°C, with detection at 350 nm. For the development of the method, different solvent systems were assayed in gradient conditions using methanol, acetonitrile and acidified water (0.5% acetic acid), at 1.0 ml/min. The best gradient was chosen: acidified methanol (0.5% acetic acid) (A) with acidified water (0.5% acetic acid) (B) as follows: 10 – 50% A (0 – 5 min), 50% A (5 – 10 min), 50 – 80% A (10 – 15 min) and 80% A (15 – 25 min).

Individual injections of ethanolic extract solution (1.0 mg/ml) were performed before all the measurements, to assess the suitability parameters, including retention time (t_R), resolution (R) between rutin and neighboring peak, capacity factor (k') and selectivity factor (α). Dried extracts were diluted at 1.0 mg/ml in methanol. The solution was filtered through a 0.45 mm membrane prior to injection into the HPLC system.

The validation of analytical procedures was performed according to the ICH guidelines (ICH, 2005).Validated parameters were specificity, linearity, accuracy, precision (repeatability and intermediate precision), limit of quantification (LOQ) and limit of detection (LOD).

DPPH Test

The free radical scavenging activities of the extracts were measured by the DPPH method. The DPPH radical (DPPH•) solution (33 µg/ml) was prepared in methanol. A volume of 30 µL of the DPPH solution was mixed with 70 µL of the tested extracts. The mixture was mixed and incubated at room temperature in the dark for 30 min. The absorbance of the samples was recorded at 570 nm against a blank, and the results were expressed as % of remaining DPPH• (Mandade *et al.*, 2011).

Nitric oxide Test

A volume of 10 µL of prediluted ethanolic extracts was incubated with 1ml of sodium nitroprusside (NANP) 5Mmfor 2 hours. A standard curve was obtained using gallic acid solution and rutin solution. A 100 µL aliquot of the tubes containing the samples was placed in the wells of the plate containing the distinct calibration curve solutions.1 ml of phosphate buffer solution (PBS) was used as blank, and 1 ml of NANP was used as control for maximum NO release. In addition1 ml ethanol was used as vehicle, to dissolve the sample. Next, 100 µL of freshly prepared Griess reagent was added to each of the wells and mixed for five

minutes. The absorbance of the samples was measured at 550 nm. The results were also expressed as % uptake of nitric oxide (Rop *et al.*, 2012).

Statistical Analysis

All determinations were carried out in triplicate. The influence of the extraction conditions was evaluated using Principal Component Analysis (PCA). Statistical analysis was performed using the "R" software.

RESULTS AND DISCUSSION

The HPLC method developed for rutin quantification proved to be linear in the range of 0.156 – 40.0 µg/ml. The correlation coefficient (r^2) was 0.9995. The limit of quantification was determined experimentally as 0.1562 µg/ml and the detection limit was 0.0078 µg/ml. The results obtained for the precision and accuracy of the method are shown in Table 2.

Table 2: Precision and accuracy of the HPLC-DAD method for rutin quantification in *P. peruviana* calyces.

Compound	Repeatability		Intermediate precision		Accuracy		
	Concentration (µg/ml)	R.S.D (%)	Concentration (µg/ml)	R.S.D (%)	Concentration(µg/ml)	Recovery(%)	R.S.D (%)
Rutin	40 2.5 0.15	1.6 1.3 0.2	2.5	2.8	2.5	102.2	0.3

R.S.D.: Relative Standard Deviation

The total phenolic contents observed in this study for the 12 extracts prepared from the calyces (ranged from 11.99 ± 0.04 to 20.86 ± 0.36 mg gallic acid/g of extract) are lower than those previously reported for *P. peruviana* leaves (Wu *et al.*, 2006). In that work, the authors found that the total phenolic content, according to the extraction solvent used, was higher with supercritical CO_2 and ethanol 95%, and lower with boiling water (90.80, 88.81,19.64 G.A./g extract, respectively).

Antioxidant activity was evaluated using two different in vitro assays; uptake of nitric oxide, a biologically important reactive oxygen species, and DPPH• scavenging activity, a mechanistic test. The estimated values of DPPH• scavenging activity ranged from 25.4± 0.5 to 60.2±1.2%, while nitric oxide uptake activity ranged from 7.1 ± 1.7 to 68.7 ± 1.1, as described in Table 3. A recent study about the effects of cultivar and harvest time on fruits of *P. peruviana* reported DPPH• scavenging activity range from 18.47 to 26.56% inhibition (Bravo *et al.*, 2015). Vilaplana and co-workers (2014) reported higher DPPH• scavenging activity for calyces than for fruits of *P. peruviana* (4.49 mmol and 1.60 Trolox/100 g of extract, respectively). On the other hand, although there is no reported data on nitric oxide uptake specifically for *P. peruviana* calyces, a previous work carried out with an ethanolic extract of the fruits found nitric oxide uptake activity of 35.02% (Rop *et al.*, 2012), close to that obtained in the present study. Since previous investigations have reported that rutin is the major compound of calyces of *P. peruviana*, and is responsible of its antioxidant activity (Toro *et al.*, 2014;Vilaplana *et al.*, 2014), it was important in this work to develop an HPLC-DAD for rutin quantification in *P. peruviana* calyces. The values of rutin ranged from 5.12 ± 0.03 to 13.25 ± 0.02 µg rutin/mg extract (Table 3). These values are higher than those previously reported (1.47 µg rutin/mg extract) for methanolic extracts of calyces of *P. peruviana* (Vilaplana *et al.*, 2014).

Table 3: Yield, antioxidant activity, total phenolic content and rutin content of ethanolic extracts of calyces of *P. peruviana*.

Extract	Yield (%)	DPPH• scavenging (%)	Nitric oxide uptake (%)	Total phenolic content (mg G.A./g extract)	Rutin content (µg/mg extract)
1	22	45.7 ± 2.8	47.7 ± 1.5	20.86 ± 0.3	13.25 ± 0.0
2	13	30.0 ± 2.5	23.2 ± 0.6	16.85 ± 0.1	6.42 ± 0.0
3	13	29.8 ± 1.5	21.4 ± 1.6	17.14 ± 0.2	5.74 ± 0.1
4	20	60.2 ± 1.2	27.9 ± 1.7	17.15 ± 0.3	5.88 ± 0.0
5	23	45.8 ± 2.0	46.9 ± 0.4	20.44 ± 0.3	6.42 ± 0.1
6	23	36.8 ± 3.7	51.4 ± 1.4	18.66 ± 0.5	8.33 ± 0.2
7	15	30.1 ± 1.5	58.4 ± 1.3	16.80 ± 0.1	6.52 ± 0.0
8	20	44.9 ± 0.8	37.7 ± 1.7	14.51 ± 0.2	13.00 ± 0.0
9	12	26.7 ± 0.5	63.3 ± 1.3	17.15 ± 0.2	7.41 ± 0.0
10	12	25.4 ± 0.5	68.7 ± 1.1	16.37 ± 0.3	5.12 ± 0.0
11	21	46.4 ± 3.0	7.1 ±1.7	11.99 ± 0.0	10.81 ± 0.0
12	14	32.1 ± 2.0	9.2 ± 0.1	15.52 ± 0.1	5.72 ± 0.0

Data are expressed as the mean ± standard deviation (n=3)

According to the PCA statistical analysis (Figure 1), the first group (cluster 1, which includes extracts 9, 7, 2, 3 and 12) contained the extracts with the lowest levels of rutin (5.12 µg/mg extract), DPPH• scavenging (25.4%) and extractive yield (15%). This cluster is composed wholly of extracts obtained with 96% ethanol.

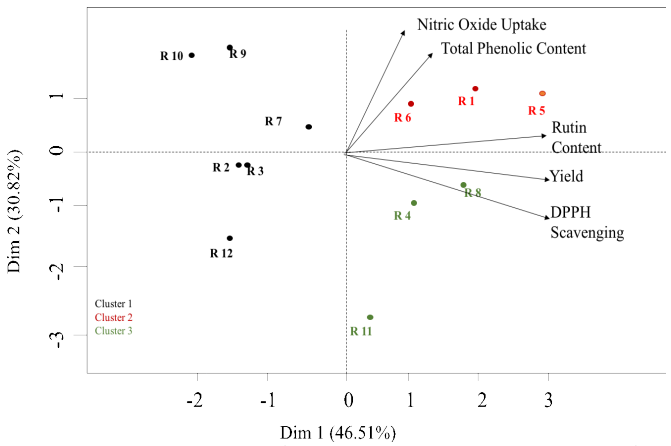

Fig. 1: Graphical illustration of the Principal Component Analysis of the extractive conditions on the yield,antioxidant activity total phenolic and rutin content of *P. peruviana* calyces extracts.

The second group (cluster 2: extracts 6, 1 and 5) includes the extracts with the highest content of total phenols (20.86 mg gallic acid/g) and rutin (13.25 µg/mg extract), all of which were

obtained with 70% ethanol and 72 hours percolation time. Finally, cluster 3 (extracts 4, 8 and 11) is characterized by extracts with high DPPH• scavenging uptake. None of the extraction conditions was found to have a significant influence, and none of them was found to be a common factor among individuals belonging to this cluster (Figure 1).

According to the results of the present work, the choice of extraction solvent was a determining factor in the extraction process. This fact could be explained by the polarity of 70% ethanol, which is most similar to the polarity of the compounds present in the extracts of calyces of *P. peruviana*. This characteristic reflected the higher antioxidant activity, total phenolic content and rutin content, according to previous reports that indicate that antioxidant activity is dependent on the solvent used in the extraction process (Ghafoori *et al.*, 2014).

Similar results have previously been reported, indicating the efficiency of ethanol (50-60%) in the extraction of rutin and total phenolic content (Kim *et al.*, 2005; Soto *et al.*, 2014). The results of this study also agree with those of other studies, which determined that the extraction solvent is the key to success in the extraction of rutin from *Hibiscus mutabilis*, according to the principle of "like dissolves like." In this study, best results were achieved with the higher polarity solvents (Xie *et al.*, 2011). A percolation time of 72 hours was the one that obtained the highest results for total phenolic content and rutin content. These results are consistent with previous studies that describe the influence of extraction time on the total phenolic content and the rutin content obtained from coffee grounds (Mussatto *et al.*, 2011). The presence of light was not a determining factor for any of the response variables evaluated. Regarding to the possible relationship between the studied variables, these are graphically illustrated in the circle of correlations, where the most closely correlated variables show a slight angle between them (Figure 2).

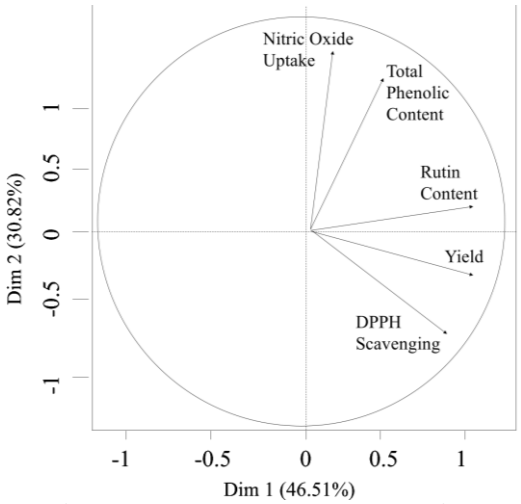

Fig. 2: Circle of correlations between evaluated variables of ethanolic extracts of calyces of *P. peruviana.*

About the results observed in Figure 2, a very good correlation was found between nitric oxide uptake and total phenolic content. These data are in accordance with the results reported previously for ethanolic extracts of *P. peruviana* fruits, which found a high correlation of $r^2 = 0.9232$ between these two variables (Rop *et al.*, 2012).

This suggests that the phenolic compounds of the extract of calyces of *P. peruviana* are responsible, at least in part, for the antioxidant activity evaluated by the nitric oxide uptake assay. A slight correlation was also found between rutin content and DPPH• scavenging activity. This correlation is expected, taking into account works that report high DPPH• scavenging activity (90.4%) by low rutin concentration (0.05 mg/ml) (Yang *et al.*, 2008). These results suggested that the antioxidant activity of rutin can be attributed to its ability to donate hydrogen to stabilize DPPH radicals.

CONCLUSIONS

Considering the statistical analysis, the optimal extraction conditions to achieve the best results in terms of yield, antioxidant activity, total phenols and rutin content are: 70% ethanol as extraction solvent, and a total percolation time of 72 hours. Light is not a determining factor. Also, a correlation was observed between total phenol content and nitric oxide uptake, while rutin content was correlated to DPPH• scavenging activity.

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