Identification of new betalains in separated betacyanin and betaxanthin fractions from ulluco (*Ullucus tuberosus* Caldas) by HPLC-DAD-ESI-MS

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Running title: An accurate method of obtaining separated betalain fractions of ulluco.

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ABSTRACT

An improved methodology of achieving an accurate separation of pure fractions of betacyanins and betaxanthins from *Ullucus tuberosus* Caldas has been carried out. For that purpose, an in-depth chemical identification of each betalain fraction using HPLC-DAD-ESI-MS was developed. This procedure enabled to evaluate the fractionation efficiency and also identify a large number of betalains, most of which have not been described so far in this raw material: betanidin- and isobetanidin-5-*O*-(4′-*O*-malonyl-*β*-glucoside), 2-decarboxy-phyllocactin, betanidin- and isobetanidin-6-*O*-(6′-*O*-feruloyl)-*β*-glucoside (gomphrenin and isogomphrenin III), dehydro-phyllocactin and isophyllocactin, and arginine and glycine-betaxanthins (portulacaxanthin III). Moreover, the availability of pure betalain fractions by the proposed methodology permitted to establish the total betalain content and the antioxidant activity of both separated betacyanin and betaxanthin fractions of ulluco for the first time. The results suggest *Ullucus tuberosus* as an underutilized food bioactive source with a high concentration of total betaxanthins (21.8 µg indicaxanthin/g fresh ulluco) and betacyanins (44.5 µg betanin/g fresh ulluco), strongly correlated to the Folin-Ciocalteau reduction capacity.

KEYWORDS

Ullucus tuberosus; betacyanins; betaxanthins; separated fractions; HPLC-DAD-MS.

INTRODUCTION

Ulluco (*Ullucus tuberosus*) is a plant endemic to the Andean region of South America, whose genus, *Ullucus*, belongs to the *Basellaceae* family. The stems of the plant are erect and later sprawling, and its height varies from 30 to 80 cm. The tubers are streaked and fleshy, with a diameter around 1 to 6 cm, and have a smooth bright surface and shallow buds. The shape could be round, cylindrical, or twisted. The skin has a wide variety of

colors, ranging from yellowish-white to magenta, through a wide range of tonalities such as yellow-green, yellow, orange, or pink [1].

Betalains are water-soluble compounds occur in a limited number of families of the order Caryophyllales and the genus *Amanita* of the Basidiomycetes. Their basic structure comprises a unit of betalamic acid that is condensed via the 3,4-dihydroxy-phenylalanine cycle (DOPA) and hydroxycinnamic acid derivatives and sugars, or amines and amino acids residues, thus leading to betacyanins (red/magenta) or betaxanthins (yellow/orange), respectively [2]. Non-specific solvents are commonly used for extracting betalains, thus the resulting extracts could contain other compounds besides betalains. Consequently, to achieve exclusively betalain-rich extracts, separation methods should be optimized. In this sense, the solid-phase extraction (SPE) column packed with C_{18} reversed-phase material is a common technique to purify betalain fraction [2], although other techniques that remove non-desirable compounds have been also applied. That is the case of Sánchez-Gonzalez et al. [3], who demonstrated that ionic exchange resins, particularly Amberlite, could be useful in the fractionation and separation of this type of pigments by removing proteins, mucilages, and pectins of the pulp of the *Opuntia joconostle*. Sawicki et al. [4] employed different stationary phases and dispersive solid-phase extraction for the determination of betalains in red beetroot, advising the use of silica-based sorbent SAX. However, scarce scientific literature about obtaining separated fractions of both betalain families has been previously reported. In that way, Stintzing et al. [2] and Betancourt et al. [5] achieved separated fractions of betacyanins and betaxanthins from the fruit of *Opuntia ficus-indica* and *Opuntia dillenii*, respectively, prior precipitation of hydrocolloids and proteins. Nevertheless, despite the promising results, these methodologies did not attain optimal accuracy and no pure separated fractions were obtained, thus improved separation methods of both betalain families are

being still needed. Particularly in ulluco, the scarce reports about its betalain characterization have been carried out using non-specific betalain solvents or purification methods [6, 7]. Therefore, no assays on purified betalain fractions isolated from *Ullucus tuberosus*, that could permit an in-depth characterization and their availability for further analyses, have been conducted until now. The development of these methodologies could permit the evaluation of the antioxidant activity of both fractions of ulluco, being only reported on extracts obtained by using non-specific solvents [6, 8-10].

An improved methodology of purification and fractionation from *Ullucus tuberosus* Caldas extracts, that permit an accurate separation of betacyanins and betaxanthin fractions, were conducted. An in-depth betalain identification of each fraction by HPLC-DAD-ESI-MS was undergone to evaluate the efficiency of the proposed methodology. The study was completed with the evaluation of the Folin-Ciocalteau reduction capacity and antioxidant activity of each pure betalain fraction to evaluate this food stock as a source of bioactive compounds.

MATERIAL AND METHODS

Plant material

The samples were collected from the village of Catambuco (Nariño, Colombia), which is located approximately 10 km south of the city of San Juan de Pasto. It is located at 1° 7′ 23″ latitude N and 77° 18′ 53″ longitude W at an altitude of 3215 m above sea level, with an average temperature of 11°C. A specimen of the plant is stored in the herbarium of the Universidad de Nariño (code number 456489). Using a simple random sampling model, in a 20 m^2 cultivation area, a representative set of samples (mature tubers of similar size with an average weight of 4.4 kg) was harvested. Only 1.2 kg of the tuber rind (magenta color) were considered, which was washed, dried, and kept at 4°C until analysis.

Preparation of the extracts

Initially, the tuber skin (magenta color) was manually removed and cut into pieces (1 cm^2). The crude fraction (CF) of the ulluco was obtained using methanol:water (60:40) for macerating the skin (2.5 mL/g sample). The procedure was repeated until the complete discoloration of the raw material. Subsequently, the organic solvent was removed at 35 ºC using a rotary evaporator (Heidolph, Schwabach, Germany), and the extract was re-dissolved with distilled water (1 g/mL) and then lyophilized (Labconco, MO, USA) [5].

Separation of hydrocolloids and proteins

CF (20 g) was dissolved with 20 mL of water and 40 mL of 96% ethanol. After 20 min of stirring, the mucilages were separated from the aqueous phase with a filter paper (Whatman No. 1 filter paper) [2]. Subsequently, the organic solvent was evaporated at 30°C using a rotary evaporator (Heidolph, Schwabach, Germany). The purified fraction (PF) was then lyophilized and kept at 4°C until analysis.

Betalain fractionation

Fractionation based on the hydrophobicity was carried out by column chromatography. Previously, a column (15 × 1.1 cm^2) with 25 g of C_{18} (Sigma-Aldrich, USA) was conditioned bypassing, separately, three volumes of 100% methanol and then three volumes of acidic deionized water with formic acid (pH 3) [5]. Subsequently, the sample (0.3 g PF / 1 mL deionized water) was added to the column. Two fractions were eluted based on their color: (a) the yellow fraction (YF, less hydrophobic compounds), using three volumes of water acidified with formic acid (pH 3), and (b) the magenta fraction (MF), with four volumes of a mixture of acetone:acidified water (60:40, v/v). Both fractions were concentrated under vacuum and then lyophilized.

Analysis of betalains by HPLC-DAD-ESI-MS

A Shimadzu LC-MS 2010 chromatographic system equipped with a quaternary pump, a UV-vis diode array detector coupled to a PC running Shimadzu LC-MS software

(Shimadzu, Tokyo, Japan) was used for HPLC separation and identification of betalains [5]. Once samples were filtered (0.45 µm nylon filter, Sigma-Aldrich, USA), betalains were separated using a Kinetex Phenomenex C_{18} column (150 × 2.1 mm, 2.6 µm particle size) maintained at 25°C, using 1% formic acid in water (v/v, eluent A) and a mixture of acetonitrile:water: formic acid (80:19:1) (eluent B) (Sigma-Aldrich, St. Louis, MO, USA). The flow rate was 0.4 mL/min, and the injection volume was 30 µL. The UV-Vis detection was carried out at 480 and 538 nm for betaxanthins and betacyanins, respectively. The identification of the individual betalains was performed by mass spectrometry (Shimadzu LC 2010, Tokyo, Japan), in positive mode using a sweeping range of *m/z* 50 to 1000. 4.5 mL/min of nitrogen was used as drying gas, the temperature was set at 250°C, and the detector voltage was 1.8 kV.

Quantification of betalains by spectrophotometry

Spectra were recorded in triplicate within the range 360-800 nm by spectrophotometry (Merck, Spectroquant® Pharo 300, USA) [5]. Two absorbances at maxima (480 and 538 nm) were reported for the quantification of betaxanthins and betacyanins, respectively. The betalain content (B) was determined using the following equation: [B] (mg/g) = [(Abs) (DF)(V)(MW)/(ε)(L)(W), where *Abs* is the maximum absorbance value at 480 or 538 nm, DF is the dilution factor, *V* is the volume (mL) of the extracts, *Mw* and ε are the molecular weight and the molar extinction coefficient of betanin (550 g/mol and 60.000 L/(mol cm) in H_2O) and indicaxanthin (308 g/mol and 48.000 L/(mol cm) in H_2O), as representative betacyanins and betaxanthins, respectively, *L* is the optical path length (0.2 cm) and *W* is the weight of the fresh sample (g). All analyses were performed in triplicate.

Folin-Ciocalteau reduction capacity (FCRC)

Methanolic extract (100 µL) was added to 900 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA). Once maintaining the solution for 5 min at room

temperature, 750 µL of sodium bicarbonate solution was added. After stirring and standing for 90 min at room temperature, the absorbance at 765 nm was measured with a spectrophotometer (Merck, Spectroquant® Pharo 300, USA) [11]. Results were expressed as mg gallic acid / g fresh weight (Sigma-Aldrich, St. Louis, MO, USA).

Determination of antioxidant capacity (TEAC)

The antioxidant capacity was measured *in vitro* based on the ability to scavenge the ABTS $^{+}$ radical, which was produced by the oxidation of 7 mM of ABTS with 2.45 mM of potassium persulfate in water. The $ABTS^{\cdot+}$ solution was diluted with phosphate-buffered saline (PBS) and adjusted to pH 7.4 after storage under dark conditions and room temperature for 16 h until reaching an absorbance of 0.70 ± 0.02 at 734 nm. Subsequently, 30 µL of each sample was added to 3 mL of the diluted solution of ABTS $^{+}$. After stirring for 1 min and waiting for 6 min, the absorbance at 734 nm was spectrophotometrically measured (UV-Vis Pharo, Merck, Germany) [12]. Results were obtained by interpolation of the absorbance in the Trolox calibration curve (0.5-3.0 µM).

Statistical analysis

All statistical analyses were performed using Statgraphics Centurion 16.1.15 software. Univariate analysis of variance (ANOVA) was applied. The mean values of each sample set (n = 3) were compared with the multiple range test at a significance level of $p < 0.05$.

RESULT AND DISCUSSION

Betalain identification and quantification

Fifteen compounds were identified in MF and six in YF (Fig. 1). The compounds identified in MF consisted of betacyanins. The presence of betanidin-5-*O*-(6′-*O*-malonyl)-β-glucoside (phyllocactin) and its isomer isobetanidin-5-*O*-(6′-*O*-malonyl)-β-glucoside (isophyllocactin) (compounds **4** and **4'**) was confirmed. For these compounds, a pseudomolecular ion was observed at m/z 637.15 units $[M+H]^+$ and daughter ions were

detected at 593.00 and 507.05 *m/z* units (Table 1). The fragment at 593.00 *m/z* units appears to have been generated by the breakdown of a carbon dioxide bond (loss of a fragment with 44 *m/z* units, corresponding to CO_2) ($[M+H-CO2]^+$) (Table 1), and the fragment at *m/z* 507.05 units could be attributed to the loss of the malonyl group $[M+H-CO_2-C_3O_3H_2]^+$. The mass spectrum also revealed a product ion at 211.95 *m/z* units, which could correspond to the betalamic acid [13]. This assignation could be confirmed by the presence of an additional absorption peak at λ_{max} 330 nm, characteristic of acyl groups. As evidenced in Fig. 1a, the major compound in this ulluco variety was phyllocactin (**4**), which represented 32.3% of the total area, whereas its isomer isophyllocactin (**4'**) represented the 8.20%. Although Svenson et al. [7] identified them in a New Zealander variety of ulluco, they reported betanin and isobetanin as the major compounds. Therefore, the major betalains could be used as a useful tool for the authenticity and origin aspects of ulluco. Phyllocactin and isophyllocactin have also been found in cacti fruits, flowers [14] and purple chard [15]. Besides, further major betalains found in ulluco were betanin (betanidin-5-*O*-β-glucoside) and isobetanin (isobetanidin-5-*O*-β-glucoside) (**1** and **2**), which represent 9.1 and 4.7% of the total area, respectively (Table 1), with a betanin/isobetanin ratio parallel to that Montes-Lora et al. [6] reported. These isomers displayed a pseudomolecular ion $[M+H]^+$ at 551.25 *m/z* units and a daughter ion at 389.00 *m/z* units due to the loss of a glucose unit $[M+H-162]^+$. Regarding gomphrenin derivatives, the isomers betanidin-6-*O*-(6′-*O*-feruloyl)-β-glucoside (gomphrenin III) and isobetanidin-6-*O*-(6′-*O*-feruloyl)-β-glucoside (isogomphrenin III) (**9** and **9'**, Table 1, Fig. 1a) were also tentatively identified, based on their similar λ_{max} (547 nm) and molecular ions (727.15 *m/z*). Although they have been already detected in *Gomphrena globosa* L. and *Bougainvillea sp* [16], their presence in *Ullucus tuberosus* has been reported for the first time in the present work.

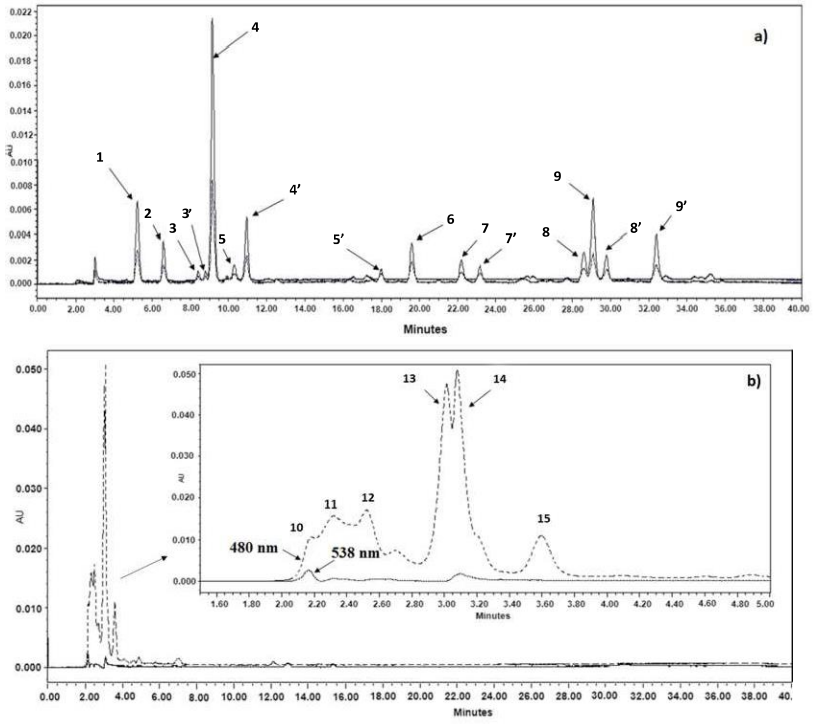

Fig. 1

This occurrence is of special relevance because gomphrenin III was the second major betacyanin in this variety of ulluco, contributing to the specificity of this raw material. This pair of compounds was distinguished from other isomers with the same values of *m/z*, such as amaranthin/isoamaranthin (betanidin/isobetanidin-5-*O*-*β*-glucuronosyl-glucoside) or lampranthin II/isolampranthin II (betanidin/isobetanidin-5-*O*-(6'-*O*-feruloyl)-*β*-glucoside) based on their t_r and λ_{max}. The more polar amaranthins elute before betanins, whereas lampranthin II and gomphrenins III do it at similar t_r. However, the location of the feruloyl-*β*-glucosyl group in position 6 of betanidin produces a bathochromic effect in the λ_{max} of gomphrenins III, which enables them to be differentiated from lampranthin II [16, 17]. Moreover, other minor isomers (**3** and **3'**), with a pseudomolecular ion at 619.15 *m/z* units $[M+H]^+$ and a fragment ion at 457.05 *m/z* units ascribed to one moiety of hexose (glucose) $[M+H-162]^+$, were tentatively described as the dehydrated form of phyllocactin and isophyllocactin, and have been firstly identified in *Ullucus tuberosus* Caldas. Other acylated betacyanins structures were also

identified in MF (**5** and **5'**) (Table 1, Fig. 1a), which exhibited a pseudomolecular ion $[M+H]^+$ similar to that of compounds **4** and **4'** (λ_{max} of 534 nm, 637.15 *m/z* units, and an ion fragment at 507.05 *m/z* units). Peaks **5** and **5'** were tentatively assigned as betanidin-5-*O*-(4′-*O*-malonyl-*β*-glucoside) and isobetanidin-5-*O*-(4′-*O*-malonyl-*β*-glucoside), respectively (Online Resource 1). Although these compounds have been previously described in *Hylocereus* species [18], this is the first attempt to identify them in ulluco, together to the compound **6,** tentatively assigned as the product of the decarboxylation of phyllocactin (2-decarboxy-phyllocactin, 593.00 *m/z* units). As aforementioned commented, these findings also could contribute to the typicality of the ulluco varieties. Furthermore, the mass data of the minor pigments **7** and **7'** enabled us to tentatively identify them as betanidin-feruloyl-5-*O*-*β*-diglucoside and isobetanidin-feruloyl-5-*O*-*β*-diglucoside, respectively (889.55 *m/z* units) (Table 1) [7]. Also, based on their spectral characteristics, pigments **8** and **8'** (λ_{max} 540 nm) were identified as isomers of lampranthin II [8]: betanidin-5-*O*-(6′-*O*-feruloyl)-*β*-glucoside and isobetanidin-5-*O*-(6′-*O*-feruloyl)-*β*-glucoside, respectively (727.15 *m/z* units and a fragment ion at *m/z* 683.55 units due to the loss of a CO_2 unit $[M+H-44]^+$). Overall, the lack of betaxanthins in MF evidenced that the proposed methodology permitted to obtain a pure betacyanin fraction, a fact that made possible to identify a high number of betacyanins in ulluco, some of them not previously reported in this raw material.

Similarly, the absence of outstanding signals at 538 nm in YF (Fig. 1b) also indicates that this fraction is deprived of betacyanins, confirming the accurate separation of both betacyanins and betaxanthins fractions by the proposed methodology. Among the betaxanthins identified, as can be seen in Table 1, pigments **13**, **14** and **15** were the major compounds in YF. Compound **14** (λ_{max} 470 nm, 368.00 *m/z* units) was assigned as arginine-betaxanthin, and compound **13** as threonine-betaxanthin (313.15 *m/z* units).

Table 1. Molecular formula, retention times (t_r), % area, UV-Vis data and mass spectral results for betalains identified in *Ullucus tuberosus* by HPLC-DAD-ESI-MS.

Peak No.	Compounds	Molecular formula	t_r (min)	Area %	UV–vis maximum (nm)	*m/z* $[M + H]^+$	MS ions
Magenta fraction							
Betacyanins							
1	Betanin-5-*O*-*β*-glucoside (betanin)	$C_{24}H_{26}N_2O_{13}$	5.6	9.1	535	551.25	389.00
2	Isobetanin-5-O-β-glucoside (isobetanin)	$C_{24}H_{26}N_2O_{13}$	6.9	4.7	535	551.25	389.00
3	Dehydro-phyllocactin	$C_{27}H_{26}N_2O_{15}$	8.5	1.1	538	619.15	457.05
3'	Dehydro-isophyllocactin	$C_{27}H_{26}N_2O_{15}$	8.8	1.0	537	619.15	457.05
4	Betanidin-5-*O*-(6′-*O*-malonyl)-*β*-glucoside (phyllocactin)	$C_{27}H_{29}N_2O_{16}$	9.1	32.3	535	637.15	593.00, 507.05, 211.95
5	Betanidin-5-*O*-(4′-*O*-malonyl-*β*-glucoside)	$C_{27}H_{28}N_2O_{16}$	10.1	1.8	535	637.15	507.05, 211.95
4'	Isobetanidin-5-*O*-(6′-*O*-malonyl)-*β*-glucoside (isophyllocactin)	$C_{27}H_{29}N_2O_{16}$	10.8	8.2	535	637.15	593.00, 507.05, 211.95
5'	Isobetanidin-5-*O*-(4′-*O*-malonyl)-*β*-glucoside	$C_{27}H_{28}N_2O_{16}$	17.9	1.0	535	637.15	507.05, 211.95
6	2-Decarboxy-phyllocactin	$C_{26}H_{28}N_2O_{14}$	19,6	5.2	533	593.00	507.00
7	Betanidin-feruloyl-5-*O*-*β*-diglucoside	$C_{40}H_{45}N_2O_{21}$	22.2	2.8	533	889.55	211.90
7'	Isobetanidin-feruloyl-5-*O*-*β*-diglucoside	$C_{40}H_{45}N_2O_{21}$	23.2	2.0	532	889.55	211.90
8	Betanidin-5-*O*-(6′-*O*-feruloyl)-*β*-glucoside (lampranthin II)	$C_{34}H_{34}N_2O_{16}$	28.6	4.2	540	727.15	683.55
9	Betanidin-6-*O*-(6′-*O*-feruloyl)-*β*-glucoside (gomphrenin III)	$C_{34}H_{34}N_2O_{16}$	29.0	12.9	547	727.15	[a]
8'	Isobetanin-5-*O*-(6′-*O*-feruloyl)-*β*-glucoside (isolampranthin II)	$C_{34}H_{34}N_2O_{16}$	29.8	4.0	540	727.15	683.55
9'	Isobetanin-6-*O*-(6'-*O*-feruloyl)-*β*-glucoside (isogomphrenin III)	$C_{34}H_{34}N_2O_{16}$	32.3	7.1	547	727.15	[a]
Yellow fraction							
Betaxanthins							
10	Unknown	-	2.1	4.2	474	381.00	[a]
11	Asparagine-betaxanthin (vulgaxanthin III)	$C_{14}H_{17}N_2O_7$	2.3	7.0	457	326.05	[a]
12	Glycine-betaxanthin (portulacaxanthin III)	$C_{11}H_{12}N_2O_6$	2.5	12.6	469	269.12	[a]
13	Threonine-betaxanthin	$C_{13}H_{16}N_2O_7$	2.7	23.8	465	313.15	269.02
14	Arginine-betaxanthin	$C_{15}H_{21}N_5O_6$	2.9	24.4	470	368.00	324.05
15	Glutamine-betaxanthin (vulgaxanthin I)	$C_{14}H_{17}N_3O_7$	3.0	21.2	467	340.10	296.12

[a] Fragmentation was not achieved.

Both compounds were already described in yellow ulluco [7] and *Gomphrena globosa* [16]. Despite being the arginine-betaxanthin the major betaxanthin in the studied ulluco, it was not previously found in other red-skinned ulluco varieties, fact that could also contribute to the specificity of ulluco varieties. Moreover, compound **15** (*m/z* 340.10 units), assigned as glutamine-betaxanthin (vulgaxantin I), was already reported in *Ullucus tuberosus* [7] or *Opuntia* sp. [19]. Among the minor betaxanthins, compound **11** (*m/z* 326.05 units) was tentatively identified as asparagine-betaxanthin (vulgaxanthin III), being previously mentioned in *Ullucus tuberosus* [7], *Beta vulgaris* L. [2, 15], and *Opuntia* spp. [2, 19]. Besides, compound **12**, with a pseudomolecular ion $[M+H]^+$ at 269.12 *m/z* units, was tentatively identified as glycine-betaxanthin (portulacaxanthin III) [15] and identified in this study for the first time.

Betalain content, Folin-Ciocalteau reduction capacity and antioxidant activity

Once evaluating the efficiency of the proposed fractionation methodology, the betalain quantification and evaluation of the Folin-Ciocalteau reduction capacity (FCRC) and antioxidant activity (TEAC) of separated pure betacyanin and betaxanthin fractions was described in ulluco for the first time.

Regarding the quantitative terms, the total content of betalains in the tuber skin was 66.3 ± 0.8 µg/g. The betacyanins were the major significant fraction ($p < 0.05$) in comparison with that of the betaxanthins (44.5 ± 0.8 µg of betanin/g and 21.8 ± 0.6 µg of indicaxanthin/g, respectively). To compare with other authors, Campos et al. [8] previously reported a betacyanin concentration of 64 µg/g and a betaxanthin amount that ranged between 22-96 µg/g. Also, Svenson et al. [7] and Cejudo-Bastante et al. [20] found total betalain concentration in *Ullucus tuberosus* in a similar range (38-80 µg/g). To establish a comparison with other sources of betalains, *Beta vulgaris* L. ssp presented a similar concentration of total betalains [15], whereas Butera et al. [21] and Gasztonyi et

al. [22] found higher betalain content in Sicilian prickly pear (*Opuntia ficus-indica*) and *Beta vulgaris* var. conditiva (90 and 500 µg/g, respectively). Anyway, *Ullucus tuberosus* represents an important source of betalains compared to other fruits or tubers.

Table 2 shows the data of FCRC and TEAC of the different fractions of *Ullucus tuberosus*. The TEAC value of ascorbic acid, the positive control, was in agreement with other authors [12]. The results displayed significant ($p < 0.05$) differences of antioxidant activity and FCRC among all the samples isolated during the purification process (CF, PF, MF, YF) (Table 2). The higher content of CF against PF could be due to the removal of some antioxidant compounds during the purification process. Notably, MF showed significantly ($p < 0.05$) higher values of TEAC and FCRC than YF, exhibiting the fraction rich in betacyanins (MF) a greater antioxidant activity and phenolic content than the fraction rich in betaxanthins (YF).

Table 2. Mean values and standard deviation ($n = 3$) of Folin-Ciocalteu reduction capacity (FCRC) (mg gallic acid/g tuber) and antioxidant activity (TEAC) (µmol Trolox/g tuber) of each separated fraction of *Ullucus tuberosus*.

	FCRC		TEAC	
CF	15.83 ± 0.39	a	97.15 ± 10.28	a
PF	12.09 ± 0.25	b	80.25 ± 6.36	b
YF	5.95 ± 0.14	c	51.55 ± 3.53	c
MF	267.17 ± 6.76	d	1387.52 ± 177.91	d

CF = crude fraction, PF = purified fraction, YF = yellow fraction, MF = magenta fraction. *Different letters in the same column denote significant differences ($p < 0.05$) among samples.

These findings were in agreement with Butera et al. [21], who affirmed that pure betanin is more active than indicaxanthin in terms of radical-scavenging capacity. Moreover, it was found that the anti-radical activity (TEAC) is consistent with the FCRC data when a univariate linear correlation is applied, observing a very strong correlation between them ($R^2 = 0.9988$), similarly to that previously reported Moussa-Ayoub et al. [23] and Betancourt et al. [5] in *Opuntia dillenii* fruits. This is the first attempt to evaluate the

antioxidant activity of pure fractions of betacyanins and betaxanthins from ulluco, revealing that this raw material is a remarkable source of bioactive compounds.

CONCLUSIONS

The fractionation methodology described here permitted to obtain separated highly-purified fractions of betacyanins and betaxanthins, improving the scarce and deficient separation methods reported up to the present. This efficiency was demonstrated by an accurate identification of betalains, which showed the absence of betacyanins in the yellow fraction and vice versa. The fact of having available pure fractions permitted to (i) identify a high number of betacyanins and betaxanthins, some of them tentatively identified for the first time in this raw material: betanidin-5-*O*-(4′-*O*-malonyl-*β*-glucoside) and isobetanidin-5-*O*-(4′-*O*-malonyl-*β*-glucoside), 2-decarboxy-phyllocactin, dehydro-phyllocactin, dehydro-isophyllocactin, betanidin-6-*O*-(6′-*O*-feruloyl)-*β*-glucoside and isobetanidin-6-*O*-(6′-*O*-feruloyl)-*β*-glucoside (gomphrenin and isogomphrenin III), arginine-betaxanthin and glycine-betaxanthin (portulacaxanthin III), and (ii) to evaluate the remarkable antioxidant activity and Folin-Ciocalteau reduction capacity of both separated betalain fractions (betacyanins and betaxanthins) for the first time in *Ullucus tuberosus*. Therefore, this separation methodology could permit to deepen further functions of both pure separated fractions and, on the other hand, could aid in the valorization and exploitation of this wild and undervalued food bioactive source.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The author(s) declared no potential conflicts of interest.

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(*Oxalis tuberosa* Molina) and ulluco (*Ullucus tuberosus* Caldas). J Sci Food Agric 86:1481-1488.

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FIGURE CAPTIONS

Fig. 1 Overlapping chromatographic profiles of (a) magenta fraction (MF) and (b) yellow fraction (YF), at 538 nm (continuous line) and 480 nm (dashed line).

Conflict of Interest and Authorship Conformation Form

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Figure 1

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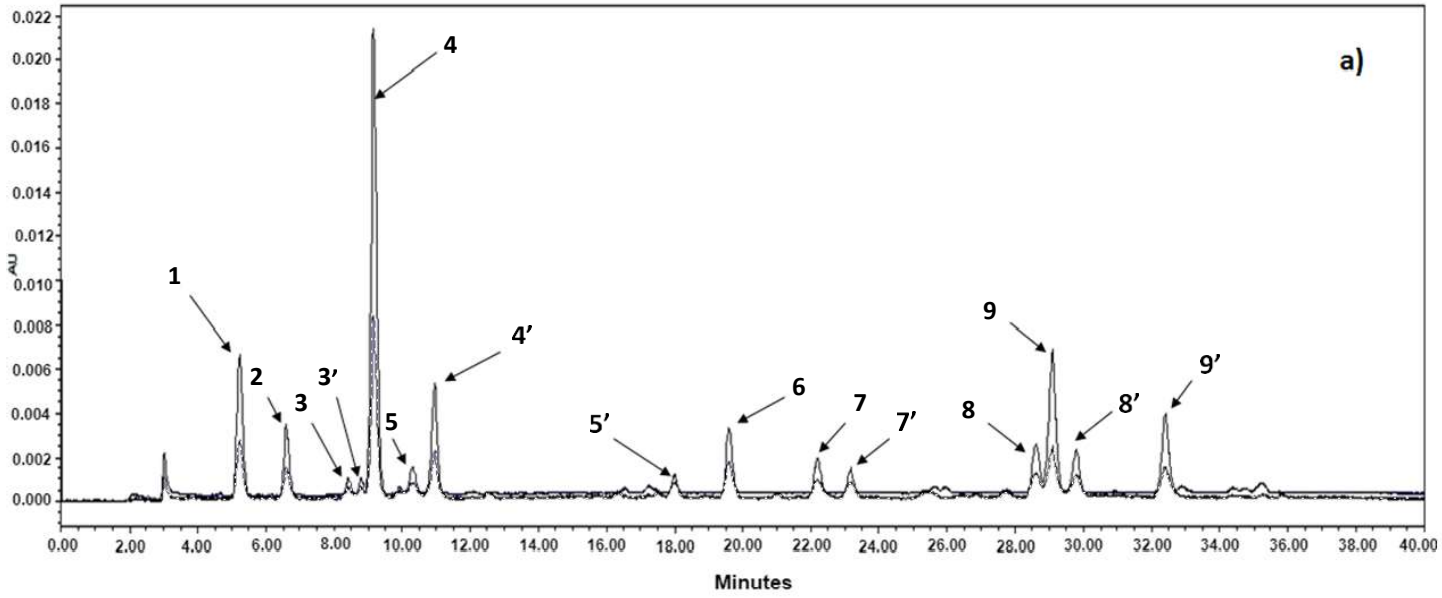


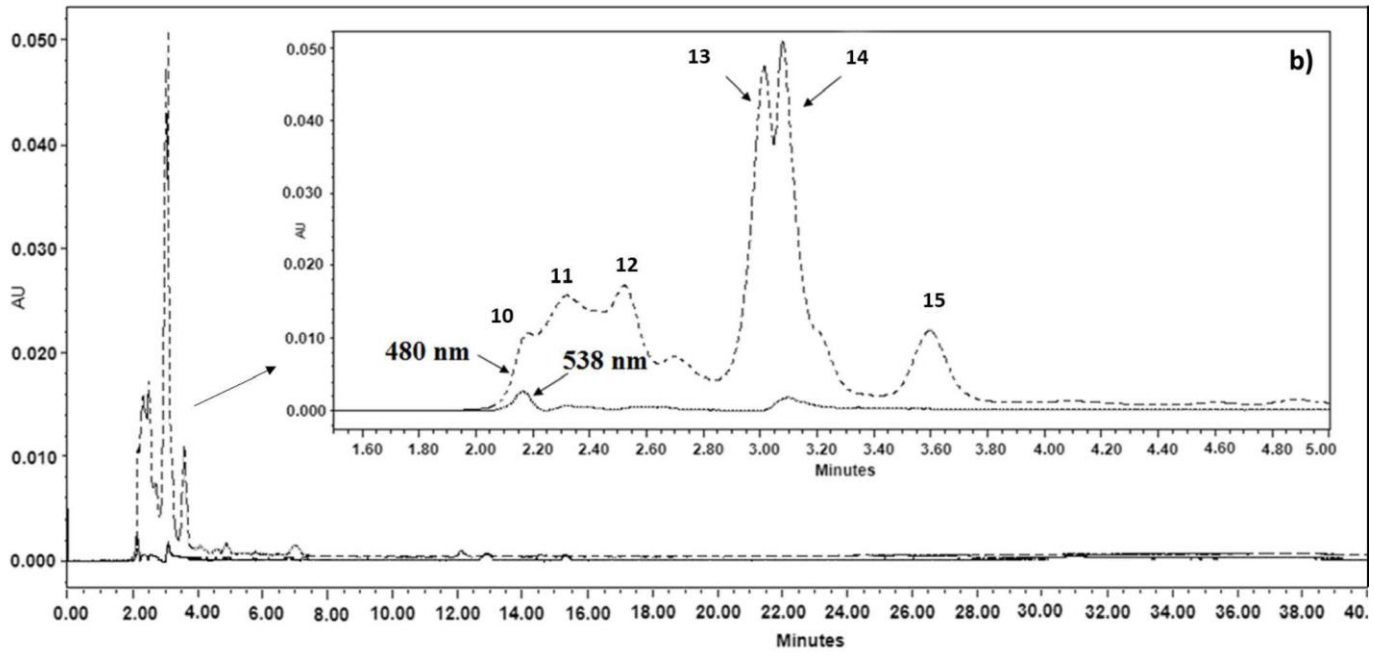


Table 1. Molecular formula, retention times (t_r), % area, UV-Vis data and mass spectral results for betalains identified in *Ullucus tuberosus* by HPLC-DAD-ESI-MS.

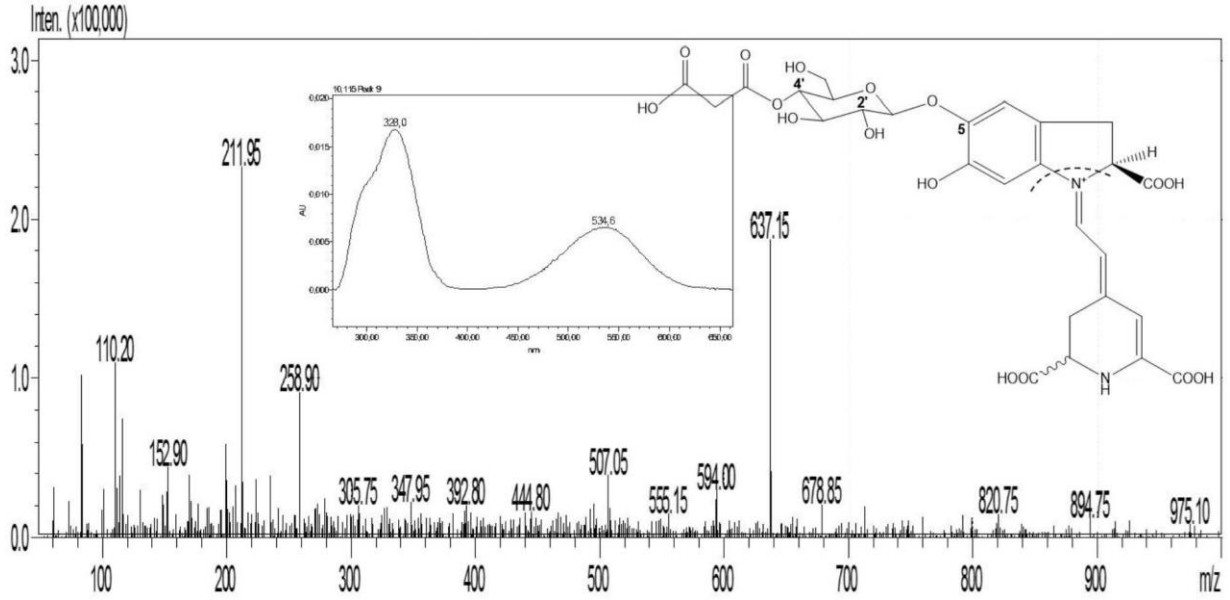
Peak No.	Compounds	Molecular formula	t_r (min)	Area %	UV–vis maximum (nm)	*m/z* $[M+H]^+$	MS ions
Magenta fraction							
Betacyanins							
1	Betanin-5-*O*-*β*-glucoside (betanin)	$C_{24}H_{26}N_2O_{13}$	5.6	9.1	535	551.25	389.00
2	Isobetanin-5-O-β-glucoside (isobetanin)	$C_{24}H_{26}N_2O_{13}$	6.9	4.7	535	551.25	389.00
3	Dehydro-phyllocactin	$C_{27}H_{26}N_2O_{15}$	8.5	1.1	538	619.15	457.05
3'	Dehydro-isophyllocactin	$C_{27}H_{26}N_2O_{15}$	8.8	1.0	537	619.15	457.05
4	Betanidin-5-*O*-(6′-*O*-malonyl)-*β*-glucoside (phyllocactin)	$C_{27}H_{29}N_2O_{16}$	9.1	32.3	535	637.15	593.00, 507.05, 211.95
5	Betanidin-5-*O*-(4′-*O*-malonyl-*β*-glucoside)	$C_{27}H_{28}N_2O_{16}$	10.1	1.8	535	637.15	507.05, 211.95
4'	Isobetanidin-5-*O*-(6′-*O*-malonyl)-*β*-glucoside (isophyllocactin)	$C_{27}H_{29}N_2O_{16}$	10.8	8.2	535	637.15	593.00, 507.05, 211.95
5'	Isobetanidin-5-*O*-(4′-*O*-malonyl)-*β*-glucoside	$C_{27}H_{28}N_2O_{16}$	17.9	1.0	535	637.15	507.05, 211.95
6	2-Decarboxy-phyllocactin	$C_{26}H_{28}N_2O_{14}$	19,6	5.2	533	593.00	507.00
7	Betanidin-feruloyl-5-*O*-*β*-diglucoside	$C_{40}H_{45}N_2O_{21}$	22.2	2.8	533	889.55	211.90
7'	Isobetanidin-feruloyl-5-*O*-*β*-diglucoside	$C_{40}H_{45}N_2O_{21}$	23.2	2.0	532	889.55	211.90
8	Betanidin-5-*O*-(6′-*O*-feruloyl)-*β*-glucoside (lampranthin II)	$C_{34}H_{34}N_2O_{16}$	28.6	4.2	540	727.15	683.55
9	Betanidin-6-*O*-(6′-*O*-feruloyl)-*β*-glucoside (gomphrenin III)	$C_{34}H_{34}N_2O_{16}$	29.0	12.9	547	727.15	[a]
8'	Isobetanin-5-*O*-(6′-*O*-feruloyl)-*β*-glucoside (isolampranthin II)	$C_{34}H_{34}N_2O_{16}$	29.8	4.0	540	727.15	683.55
9'	Isobetanin-6-*O*-(6’-*O*-feruloyl)-*β*-glucoside (isogomphrenin III)	$C_{34}H_{34}N_2O_{16}$	32.3	7.1	547	727.15	[a]
Yellow fraction							
Betaxanthins							
10	Unknown	-	2.1	4.2	474	381.00	[a]
11	Asparagine-betaxanthin (vulgaxanthin III)	$C_{14}H_{17}N_2O_7$	2.3	7.0	457	326.05	[a]
12	Glycine-betaxanthin (portulacaxanthin III)	$C_{11}H_{12}N_2O_6$	2.5	12.6	469	269.12	[a]
13	Threonine-betaxanthin	$C_{13}H_{16}N_2O_7$	2.7	23.8	465	313.15	269.02
14	Arginine-betaxanthin	$C_{15}H_{21}N_5O_6$	2.9	24.4	470	368.00	324.05
15	Glutamine-betaxanthin (vulgaxanthin I)	$C_{14}H_{17}N_3O_7$	3.0	21.2	467	340.10	296.12

[a] Fragmentation was not achieved.

Table 2. Mean values and standard deviation ($n = 3$) of Folin-Ciocalteu reduction capacity (FCRC) (mg gallic acid/g tuber) and antioxidant activity (TEAC) (μmol Trolox/g tuber) of each separated fraction of *Ullucus tuberosus*.

	FCRC		TEAC	
CF	15.83 ± 0.39	a	97.15 ± 10.28	a
PF	12.09 ± 0.25	b	80.25 ± 6.36	b
YF	5.95 ± 0.14	c	51.55 ± 3.53	c
MF	267.17 ± 6.76	d	1387.52 ± 177.91	d

CF = crude fraction, PF = purified fraction, YF = yellow fraction, MF = magenta fraction. *Different letters in the same column denote significant differences ($p < 0.05$) among samples.



Online Resource 1. Mass spectrum of pigment 6 (betanidin-5-*O*-(4′-*O*-malonyl-*β*-glucoside)

Ms. Ref. No.: QUAL-D-20-00170

Title: Identification of new betalains in separated betacyanin and betaxanthin fractions from ulluco (Ullucus tuberosus Caldas) by HPLC-DAD-ESI-MS

Reviewer: 1

Pag 4, Line 19. CF (20 g)

Corrected.

Pag 6, Line 2. Methanolic extract (100 µl) was..

Corrected.

Pag6, Line 19. ABTS.+ solution (7mM ABTS + 2.45 mM potassium persulfate) was diluted with phosphate-buffered saline (PBS)

Corrected.

Please clarified, 2.45 mM potassium persulfate was prepared as stock in water?, the same for ABTS or bot were prepared in PBS?

Corrected. Please see "Determination of antioxidant capacity (TEAC) section".

Reviewer: 2

The only new information in this manuscript is another betalain profile which is different from the profiles presented in [8] for red and yellow tubers. Here a profile for purple tubers is presented which is more rich in betacyanins but less in betaxanthins.

The preparation as well as chromatographic separation of the sample is not novel, especially the using of a column which seems to be working in a plain C-18 flash chromatography mode which is not specified - no particle diameter is mentioned. However, taking into consideration obtained fractions - just 2 fractions - no high-performance was applied. Therefore, only mixtures of betalains are obtained for further tests, so only rough estimation of their activities is performed.

The application of the results stems from several points of view. On the one hand, the importance of valuing the under-utilized *Ullucus tuberosus* tuber due to its beneficial properties as the high antioxidant capacity. With that purpose, *Ullucus tuberosus* could be incorporated into diet and be used with cosmetic, drug and medical purposes in different presentations as a beneficial source. From a chemical point of view, it could be interesting to scrutinize what pigments are responsible or contribute more to the antioxidant capacity of the *Ullucus tuberosus*. Moreover, to achieve an accurate colorimetric characterization of betacyanin and betaxanthins fractions, stability assays only due to these fractions, the obtaining of individual betacyanins and betaxanthins from pure extracts, the subsequent researches about individual behaviours, interactions between them at dual or multiple levels, etc.

For all those purposes, it is needed a purification and fractionation method to obtain pure extracts of betalains (betacyanins and betaxanthins). This paper just represents this first step, the first step towards further researches. The methodology employed permitted a correct separation and the obtaining of pure extracts of both fractions, unlike other published separation methodologies that did not permit to obtain highly-pure fractions. To bring this fact into fruition, an in-depth identification of betacyanins and betaxanthins was carried out to evaluate the efficiency of the proposed methodology. Once demonstrated the accuracy of the method, also antioxidant activity and FCRC of those pure extracts were also analyzed and established.

The particle size of the column was already specified in the manuscript (please, see page 5, line 5).

For identification of betalains, some reference compounds derived from plants of known betalain profiles should be taken into account. In the case of betaxanthin, it is possible to do a fast and easy semi-synthesis of qualitative standards which could be compared with the pigments from the analyzed samples. For some unknown betacyanin-like pigments (3, 4, 10, 11, 12, 14), additional high-resolution MS analyses (qTOF, IT-TOF) would provide molecular formulas for confirmation as well as the fragmentation pathways - especially for 10 and 11 which is doubtful.

Authors agree with the reviewer. However, this paper is a preliminary study whose main objective is to obtain concentrated and purified fractions of the two main families of betalains (betacyanins and betaxanthins), which would suppose the beginning of further applications. From those fractions, authors will develop a method for obtaining pure standards of betalains (not commercially available) by Semi-preparative Liquid Chromatography, which could permit us to deepen in the accurate identification of betalains by standards, among others. Besides, since commercial standards of betalains are not available, this method paves the way for moving forward obtaining pure individual betalains. In fact, authors are going into the subject of the synthesis of betaxanthins, isolating betalamic acid and concreting the synthesis conditions for good yields, for being used in further papers on betalains.

The numbering of betacyanins should be performed according to typical phytochemical rules, for the isoforms a postfix " ' " should be implemented with the same number as the natural isomer.

Corrected.

For Figure 1 - the x and y scales should present bigger label fonts.

Corrected.

Reviewer: 4

Paper is proposing a method to fractionate betalains, of U. tuberosus, into betacyanins and betaxanthins and presents their MS characterization. In this regard, manuscript is not properly focused: the introduction showed a good description of the necessity to improve the method of the betalains' purification, but results are neither

properly presented nor discussed; this is clearly observed in the abstract.

The main goal of this manuscript is to develop a fractionation methodology to obtain pure separated betacyanins and betaxanthin fractions. In order to confirm the efficiency, the identification of betalains in both fractions was carried out. In the results section, the absence of outstanding signals at 538 nm in YF and at 480 nm in MF (yellow fraction is deprived of betacyanins and red fraction is deprived of betaxanthins) confirmed the accurate separation of both betacyanins and betaxanthins fractions by the proposed methodology. Moreover, the fact of obtaining pure separated fractions also permitted to identify a high number of betacyanins and betaxanthins, some of them not previously reported, and the results section also refers to that matter. Besides, the antioxidant activity of both pure fractions was first described. Overall, the proposed fractionation method supposes an important advance for betalain characterization, improving the scarce and deficient separation methods of betacyanins and betaxanthins reported up to the present. In order to a better understanding and a proper focus, some parts of the manuscript have been rewritten.

In the materials and methods section, several parts are unclear.

Material & Methods section has been modified for a better understanding.

Considering the gomphrenin III and isogomphrenin III, how were identified each other isomer?

According to the chromatographic conditions used in these assays, it has been widely reported in the literature that the elution order of each pair of betalains is normally that exposed in the manuscript. However, authors have manifested that the assignation is tentative.

Betancourt C, Cejudo-Bastante MJ, Heredia FJ, Hurtado N (2017) Pigment composition and antioxidant capacity of betacyanins and betaxanthins fractions of *Opuntia dillenii* (Ker Gawl) Haw cactus fruit. Food Res Internat 101:173-179.

Slatnar A, Stampar F, Veberic R, Jakopic J (2015) HPLC-MSn Identification of Betalain Profile of Different Beetroot (*Beta vulgaris* L. ssp. vulgaris) Parts and Cultivars. J Food Sc, 80, Nr. 9.

Cejudo-Bastante MJ, Chaalal M, Louaileche H, Parrado J, Heredia FJ (2014) Betalain Profile, Phenolic Content, and Color Characterization of Different Parts and Varieties of Opuntia ficus-indica J Agric Food Chem 62:8491–8499.

Conclusions are not supported by a good discussion.

Conclusions have been modified for a better understanding.

Good selected references must be used to support the main goals of this paper.

Scarce bibliography on the betalain profile of *Ullucus tuberosus* has been published up to the present and even less on the fractionation methodology of betacyanin and betaxanthin pure fractions. Even so, some additional bibliography has been included in the manuscript.

Minor concerns

Paper is wordy and English must be improved.

A native speaker has revised English grammar throughout the text.

There are several mistakes in the references section.

The reference section has been now checked and corrected.

Figure 1 legend shows mistakes. The design of figure 1 must be improved, e.g. using a different type of lines.

Figure 1 legend and its design have been now modified.

There are two references where the antioxidant activity of this material is discussed:

Peñarrieta, J. M., Alvarado, J. Antonio, kesson, Björn, & Bergenståhl, Björn. . , . (2005). TOTAL ANTIOXIDANT CAPACITY IN ANDEAN FOOD SPECIES FROM BOLIVIA. Revista Boliviana de Química, 22(1), 89-93.

Mejía Lotero, F. M., Salcedo Gil, J. E., Vargas Londoño, S., Serna Jiménez, J. A., & Torres Valenzuela, L. S. (2018). CAPACIDAD ANTIOXIDANTE Y ANTIMICROBIANA DE TUBÉRCULOS ANDINOS (Tropaeolum tuberosum y Ullucus tuberosus). Revista U.D.C.A Actualidad & Divulgación Científica, 21, 449-456. Retrieved from http://www.scielo.org.co/scielo.php?script=sci_arttext&pid=S0123-42262018000200449&nrm=iso

There is at least one paper published in this journal and not cited (I do suggest to cite it) any of them, while you are using as reference some obscure sources.

These articles have been now mentioned in the manuscript.