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**Polyphenols bioaccessibility and bioavailability assessment in ipecac infusion using a
combined assay of simulated *in vitro* digestion and Caco-2 cell model.**

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Running title : Antioxidant potential of Ipecac infusion

Abstract:

In this report, we investigated for the first time the total polyphenols content (TPC) and antioxidant activity before and after digestion of *Carapichea ipecacuanha* root infusion, better known as ipecac, prepared at different concentrations. An *in vitro* digestion system coupled to a Caco-2 cell model was applied to study the bioavailability of antioxidant compounds. The ability of ipecac bioaccessible fractions to inhibit reactive oxygen species (ROS) generation at cellular level was also evaluated. The findings revealed that a water volume of 50 mL/g of sample provided the maximum yield of extraction of TPC and antioxidant activity. Polyphenols increased in content and activity after digestion and they were highly bioavailable (75 % of intestinal absorption). Polyphenols were also present in the residual parts which indicate a possible local activity. Results also suggest that ipecac infusion could represent a promising source of effective bioavailable antioxidants to be exploited in functional foods field.

Keywords: Ipecac infusion, polyphenols, antioxidant activity, *in vitro* digestion, Caco-2 cells, bioavailability.

Introduction

Carapichea ipecacuanha is a medicinal plant with a long history of use in traditional medicine and as an over the counter drug. The plant, belonging to the Rubiaceae family, occurs naturally in warm humid forest and it is originally native from the rainforests in central and south America (Itoh *et al.*, 1999, Júnior *et al.*, 2012). *C. ipecacuanha* medicinal importance is gained from the expectorant, emetic and antihemorrhagic properties of its reddish-brown root commonly known as ipecac and generally used as syrup or as an infusion (Garcia *et al.*, 2005). Although it has been already reported that cephaline and emetine are the major alkaloids found in ipecac in addition to psychotrine (Itoh *et al.*, 1999, Júnior *et al.*, 2012) and that it also contain tannins, ipecacuanhic acid, saponins, glycosides and cyclotides (Panda 2002; Fahradpour *et al.*, 2017), there is no sufficient data concerning its biochemical composition, especially concerning phenolic compounds.

According to literature, besides its expectorant, emetic and amebic activities (Garcia *et al.*, 2005), ipecac has shown other interesting pharmacological activities such as anti-amebic, anticancer and anti-inflammatory (Möller *et al.*, 2007, Júnior *et al.*, 2012). A scientific study even revealed that ipecac-induced emesis is more efficient in removing excess salicylate than gastric lavage (Boxer *et al.*, 1969). However, the abuse of ipecac ingestion can be harmful since an inhibition of the immune response in mice by emetine, the major alkaloid found in ipecac, has been reported (Csuka & Antoni 1984).

On the other hand, *C. ipecacuanha* has been used for a long time in traditional medicine to treat digestive disorders (especially chronic diarrhea), gastroenteritis and ulcerative colitis (Saganuwan 2010). In Tunisia, some people believe that drinking ipecac infusion is good for treating hormonal imbalance in women and thus increasing the chances of pregnancy. The beneficial applications of this infusion could be related to the presence of bioactive

compounds with antioxidant properties, such as polyphenols, as it has been shown in other herbal plants used for infusions (Jiménez-Zamora *et al.*, 2016). However, ipecac is already consumed as an infusion without investigating its composition, antioxidant activity, nor the polyphenols content and fate inside the gastrointestinal tract. These luring reasons may justify conducting a study exploring the bioavailability of phenolic compounds of ipecac as a first step for revealing more information and knowledge concerning this plant. It is a well-established fact that polyphenols are the most abundant antioxidants in the human diet and they are known for their wide variety of biological activities and for the prevention from different kind of illnesses such as cancer and cardiovascular diseases (Belhadj *et al.*, 2016, Hlel *et al.*, 2017). However, the effects of polyphenols on health are inseparable from the notion of bioavailability. In brief, the bioavailability of polyphenols can be defined as the fraction of phenolic compounds that reaches the systemic circulation and the target tissue, where it will exert a biological function after being ingested (Manach *et al.*, 2004). In order to be bioavailable, a molecule must be released from the consumed food during digestion, i.e., be bioaccessible, absorbed by the intestinal cells and transported to the tissue(s) (Rein *et al.*, 2013). Moreover, the efficacy of polyphenols depends greatly on their absorption while their bioavailability itself depends on several factors mainly the chemical structure of the phenolic compound, the food matrix, the interactions with other compounds and the host related factors (D'Archivio *et al.*, 2010). The assessment of polyphenols bioavailability can be conducted *in vivo* and/or *in vitro*. Although *in vivo* assays could offer more advantages in terms of accuracy, a wide number of researchers have used cell cultures, namely Caco-2 cells, as a useful tool to study the intestinal absorption of phenolic acids and flavonoids (Konishi *et al.*, 2003, Pérez-Sánchez *et al.*, 2017; Achour *et al.*, 2018). In fact, this colonic line has the ability of differentiating, under normal culture conditions, into a cellular monolayer resembling an intestinal epithelium mimicking a functional intestinal barrier (Lea 2015).

To the best of our knowledge, no published study exploring antioxidant potential, polyphenols content and bioavailability of ipecac infusion is available. Therefore, this study will shed some light on this subject and provide new data that can be exploited in both scientific and industrial fields regarding pharmacology and functional foods applications.

In the first part of this work, ipecac infusions were prepared. It is worth noting that the infusions concentration were chosen to be as close as possible to what the majority of people are usually using to prepare their ipecac infusion in Tunisia. Then, an *in vitro* gastrointestinal digestion was performed to mimic physiological conditions with the intention of obtaining and evaluating the bioaccessible fraction. In addition, the residual fractions were also studied for further inspection. The final step was studying the bioavailability of the bioaccessible polyphenols. Antioxidant properties of the infusions were studied before and after the *in vitro* digestion of the samples, by ABTS, DPPH and FRAP methods. Moreover, antioxidant activity after digestion was tested at the cell level, by analyzing effects on reactive oxygen species (ROS) generation in Caco-2 cells, both at basal and induced oxidation conditions.

Material and Methods

Chemicals

Double distilled deionized water deionized water was obtained from a Milli-Q purification system (Millipore, Bedford, MA). Sodium bicarbonate, sodium carbonate and hydrochloric acid (37%) were purchased from Merck (Merck, Darmstadt, Germany). Methanol, Folin–Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), pepsin, pancreatin, bile salts, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and tert-butylhydroperoxide (*t*-BOOH) were obtained from Sigma (Sigma–Aldrich,

St. Louis, MO). 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) was purchased from Fluka Chemicals (Fluka Chemicals, Madrid, Spain). Cell culture media and chemicals were provided by Sigma.

Plant material and preparation of the infusions

Carapichea ipecacuanha root was collected from Beja region in the northwest of Tunisia (36°44'N, 09°11'E) and then dried at room temperature. The plant has been identified by Dr. Chokri Messaoud, professor of genetics and plant biotechnology at The National Institute of Applied Sciences and Technology (INSAT) and a voucher specimen (LC 22-2015) was deposited at the Herbarium of INSAT. Three infusions were prepared by boiling 25 mL of Milli Q water with 0.5g, 1g and 3 g of the powdered sample for 10 min (See supplementary data S1). The obtained infusions were filtered and the samples were kept in a freezer at -80°C for further assays. The samples were named 1, 2 and 3, according with initial concentrations of 0.5, 1 and 3 g/25 mL, respectively.

Colour values

Colour measurement (**CIE** L^* , a^* , b^* parameters) was performed in the infusions using a Minolta Colorimeter (CR-400, Konica Minolta Corp., Japan) with illuminant D65. The parameter L^* is a measurement of lightness according to a grey scale, black to white, ranging from 0-100. The parameter a^* takes positive values for redness and negative values for greenness while for b^* , the values are positive for yellowness and negative for blueness. The samples were placed in a 34 mm optical glass cell and each value resulted from triplicate measurements.

The *in vitro* digestion

A two-step *in vitro* digestion model, consisting in gastric and intestinal phases, was performed according to the method described by Seiquer *et al.* (2015) with slight modifications. To mimic the gastric digestion, the pH of each infusion (10 mL) was adjusted to 2 using 1 N HCl and 313 μ L of a previously prepared pepsin/0.1 N HCl solution (160 mg pepsin/mL) were added. The mixture was maintained for 2h at 37°C in a shaking bath (110 oscillations/min). After that, the pH of the digest was increased to pH 6 with 1 M NaHCO₃ and then mixed with 2.5 mL of pancreatin and bile salts mixture (0.1 g of pancreatin and 62.5 mg of bile salts in 25 mL of 0.1 M NaHCO₃). The pH was adjusted to 7.5 with NaHCO₃ followed by a 2h incubation at 37°C and 110 oscillations/min. To interrupt the digestion process, the enzymes were inactivated by a heat treatment for 4 min at 100 °C in a polyethylene glycol bath then cooled by immersion in an ice bath. Next, the samples were centrifuged at 10000 rpm for 30 min at 4 °C (Sorvall RC 6 Plus centrifuge) to separate the bioaccessible fractions (B1, B2 and B3) and the residual fractions (R1, R2 and R3), which were stored at –80°C and kept in the dark until use. To discard any interference from the reagents in the digestion process, blanks were run simultaneously with the samples. All samples were run in triplicate.

B and R fractions were used to measure total phenolic content (TPC) and antioxidant capacity. The sample with the highest antioxidant potential was selected for Caco-2 cells assays.

Total phenol content and antioxidant activity

The TPC and antioxidant activity were measured according to the methodologies given by Seiquer *et al.* (2015) using 96-well multiwell plates and a with a Victor X3 multiwell plate reader (Waltham, Massachusetts, USA). TPC was determined by the Folin-Ciocalteu colorimetric method and the absorbance was measured at 750 nm against a standard curve of

gallic acid (0-250 mg/L). Results were expressed as mg of gallic acid equivalents (GAE) per gram of sample (powdered *Carapichea ipecacuanha* root).

The antioxidant capacity was analyzed by the DPPH, ABTS (antiradical activity) and FRAP (ferric reducing power) assays, measuring the absorbance at 520, 730 and 595 nm, respectively. Results were obtained against calibration curves of Trolox and expressed in mM (DPPH) or μ M (ABTS and FRAP) equivalents of Trolox per gram of sample.

Cell culture assays

Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) through the Cell Bank of Granada University. Prior to use in this assay, Caco-2 cells were cultured for several passages in culture flasks containing Dulbecco modified minimal essential medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), NaHCO_3 (3.7 g/L), nonessential amino acids (1%), HEPES (15 mM), bovine insulin (0.1 IU/mL) and 1% antibiotic–antimycotic solution at 37°C and in a humidified atmosphere of 5%. The medium was replaced in every two days.

Cell viability

Neutral red (NR) uptake cytotoxicity assay was performed to evaluate cell viability in order to determine the adequate DMEM-sample ratio to use for the subsequent assays. Thereby, three ratios of DMEM-bioaccessible fraction (BF) (v/v) were assayed: 1:1, 1:2 and 1:3.

The cells were seeded in 96-well microtitre plates at a density of 6×10^4 cells/well in 100 μ L of the medium then incubated for 48 hours for adhesion. The medium was discarded and 100 μ L of BF were added to cells while the control wells received FBS-free DMEM. The Caco-2 cells were harvested after 2h of incubation and the cell viability was measured by staining with NR then incubated 2h at 37 °C. After cell fixation (0.5% formaldehyde, 0.1% CaCl_2 for

30 seconds at room temperature), the plates were briefly immersed in phosphate-buffered saline to wash the cells then cell lysis was performed (50% ethanol, 1% acetic acid overnight at 4 °C). The day after, the absorbance was measured at 550 nm using a BioRad Model 550 microplate reader (BioRad, CA, USA). Results were expressed as percentage of viable cells incubated with samples from data of viable control cells ($n \geq 5$ per experiment).

Absorption assay

Prior to the absorption assay, Caco-2 cells were seeded into permeable polycarbonate filter supports (Transwell, 24 mm diameter, 4.7 cm² area, 3 µm pore size, Costar), the medium was replaced every 2 days and the cell monolayer integrity was monitored during growth and differentiation using the phenol red marker. After 21 days of culture, the cells were ready to use for the permeability test since they reached confluence and the leakage rate of phenol red was less than 2.5% per hour.

Firstly, the bicameral chambers were rinsed twice with Hank's balanced salt solution (HBSS) at 37°C. 2.5 ml of the transport buffer (130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L MgSO₄, 5 mmol/L glucose, and 50 mmol/L HEPES, pH 7) were loaded to the basolateral chamber while 1.5 ml of the diluted BF (the one with the highest viability percentage) was added to the apical chamber. The buffer from the basolateral chamber was aspirated after a 2h incubation at 37°C in a humidified air:CO₂ atmosphere, and used to evaluate the total polyphenols and the antioxidant activity transported across Caco-2 cells monolayer. Absorption was calculated as the percentage transported/well from the sample initially loaded in the apical chamber.

Reactive oxygen species (ROS) generation assay

The capacity of the samples to inhibit ROS generation was tested using the dichlorofluorescein (DCF) assay described by Borges *et al.* (2017). Cells were plated in 24-well multiwell plates at a density of 2×10^5 cells per well in 1 mL of DMEM and allowed to adhere for 48h at 37°C. Then, the cells were treated with 1 mL of the BF while DMEM was used for control wells. After 2h, the medium was aspired and 1 ml of dichloro-dihydro-fluorescein (DCFH) 100 μ M were added and the cells were left to incubate for 1 hour. The DCFH was discarded and cells were treated with either tert-butyl hydroperoxide (t-BOOH) 5 mM to induce oxidation or culture medium for the basal effect evaluation. The absorbance was obtained immediately and then at different timings at a wavelength of 485 nm excitation and 535 nm emissions at 37°C. When ROS are generated, DCFH is converted into dichlorofluorescein (DCH) and emits fluorescence that can be detected and measured using the plate reader.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD) from at least 3 parallel measurements. The analysis of variance (ANOVA) and Tukey multiple comparisons were performed with the SPSS 19.0 software (SPSS Inc.). Significance of difference was defined at 5% level ($\alpha= 0.05$).

Results and discussion

Color

Results for color measurement are reported in Table 1. The L^* is the measurement of the darkness/ lightness, so that a decrease on the L^* values indicates darkening. The data showed that I3 infusion was the darkest and that the less concentrated infusion had the lighter color. Significant variations related with the sample concentration were also found for redness (a^*)

and yellowness (b^*) color values of the infusions; the a^* value increased and the b^* value decreased with increasing initial concentration for the infusion preparation, indicating higher intensity of red and less yellow color.

The instrumental colour has been related with different conditions of herbal infusions, such as the temperature and length of time (Sun *et al.*, 2017), the storage time of the plants used to obtain infusions (Jiménez-Zamora *et al.*, 2016) or the degree of fermentation level of tea (Carloni *et al.*, 2013). Colour differences of tea infusions have been also correlated with chemical composition and even with sensory quality attributes (Liang *et al.*, 2003). Moreover, relationships between color parameters and antioxidant activity and TPC of infusions have been studied: whereas some researchers have found significant correlations (Jiménez-Zamora *et al.*, 2016, Sun *et al.*, 2017) other did not (Jing *et al.*, 2016). In the present study, color analysis showed changes in line with the variations observed for the phenolic content and the antioxidant capacity of infusions, in line with previous studies (Jiménez-Zamora *et al.*, 2016). Values of L^* and b^* varied according with TPC and antioxidant properties, whereas the a^* coordinate varied in the opposite sense, as will be discussed below. This means that infusion with the highest polyphenol content and antioxidant activity was the one most luminous and with greatest greenness and yellowness.

TPC and antioxidant activities

Results of the TPC and antioxidant properties of the infusions before and after the *in vitro* digestion (bioaccessible fractions) are depicted in Table 2. Data are expressed as the quantity of phenols or antioxidant activity extracted from each gram of plant.

Significant differences ($P < 0.05$) were observed depending on proportions plant/water when preparing infusions. Reducing the volume of water in relation to the weight of sample (50, 25

and 8.3 mL of water per gram of plant in samples 1, 2 and 3, respectively) seemed to have a negative effect in the yield of extraction for antioxidant compounds. Therefore, a minimum of 50 mL of water per gram of dry material is required for maximizing extraction efficacy in *C. ipecacuaha* infusions. Results are in agreement with the work of Da Silveira *et al.* (2014) who performed a multivariate experimental design aimed to optimize the preparation conditions for tea beverages and reported that the maximum rutin content, the primary flavonoid in this plant, was obtained when the infusion was prepared using 2 g of mate tea added to 100 mL of water, that is equivalent to 50 mL per gram.

TPC and antioxidant activity measured by DPPH, ABTS and FRAP methods have been previously investigated in tea infusions and various herbal infusions (Gorjanović *et al.*, 2012, Jiménez-Zamora *et al.*, 2016; Jing *et al.*, 2016). Also, researchers have taken a great interest in medicinal plants for their phenolic concentrations and related total antioxidant potential (Carloni *et al.*, 2013). Bibliographic data are difficult to compare, since results may be expressed referred to the liquid infusion (Jimenez-Zamora *et al.*, 2016), lyophilized infusion (Dias *et al.*, 2014) or in redissolved solutions of the dried infusions (Kogiannou *et al.*, 2013). Values of TPC from *C. ipecacuanha* root are among the highest found in the literature for herbal and medicinal infusions, similar to those found for green tea (Jiménez-Zamora *et al.*, 2016) and higher than medicinal plants such as *Clivina ferrea* and *Lagerstroemia speciosa* (Carloni *et al.*, 2013). TPC have been strongly associated with antioxidant power in many foods and herb infusions (Jimenez-Zamora *et al.*, 2016; Sun *et al.*, 2017). Accordingly, reduction of the TPC value due to decreasing water volume found in the present assay was linked to reductions of antiradical capacity (DPPH and ABTS assays) and reducing power (FRAP). However, in sample 2 the TPC was reduced by around 12% from values if sample 1, whereas, DPPH and ABTS values were reduced among 50-60% and FRAP nearly 30%,

which means that in *C. ipecacuaha* infusions phenolics do not necessarily correspond to the antioxidant response, in agreement with previous reports (Atoui *et al.*, 2005). In sample 3, drastic falls (around 80-90%) of TPC and scavenger activity were observed, whereas more than 60% of the reducing capacity was maintained, indicating that the negative effect of reducing solvent is not so deleterious for the reducing substances present in the ipecac root than for those with scavenge activity.

The first step to unravel the fate of ingested polyphenols is to determine their bioaccessibility level. In fact, the bioaccessible fraction of a food matrix could be defined as the bioactive components liberated during digestion in the gastrointestinal lumen and becoming available for intestinal absorption (Rein *et al.*, 2013). In order to evaluate the impact of the simulated digestion conditions on the phenolic content and bioactivity of samples, data from TPC, DPPH, ABTS and FRAP assays were obtained from the bioaccessible fractions (Table 2).

In line with results observed in the infusions, values of TPC and antioxidant activity were B1>B2>B3, suggesting that the water volume in *C. ipecacuanha* infusions enhances the extraction and bioaccessibility of antioxidant compounds.

Furthermore, there was a rise in the TPC content in all the bioaccessible fractions which reveals that the digestion process most likely contributed in the liberation of bioactive compounds under the effect of the digestive enzymes action, temperature and pH conditions.

The increased value of TPC after digestion was associated to similar increases (between 1-2 fold) of the antiradical activity, as measured by DPPH and ABTS methods. However, the FRAP activity of the samples decreased by 3 to 29% after digestion.

In fact, the FRAP assay principle is different than DPPH and ABTS since it does not reflect the scavenging ability of the samples against free radicals but the ability of molecules to

convert Fe (III) to Fe (II) (Hlel *et al.*, 2017). The differences in principles, mechanisms and targets could explain the different results. Moreover, it has been reported that FRAP assay may have interference problems and fluctuating readings that could affect the results (Clarke *et al.*, 2013).

Overall, there was a relative improvement of the antioxidant activity, which could be partly probably due to the increase of TPC, since a possible correlation between the amount of phenolic compounds and free radical scavenging capacity of a sample has been previously reported (Ma & Huang 2014). Another possible explanation, is that the alkaloids contained in ipecac could contribute to the antioxidant activity since it has already been shown that alkaloids exhibit potent antioxidant activity (Račková *et al.*, 2004). Our results are in agreement with other studies performed on different food samples that reported an increase in the TPC and antioxidant activity after *in vitro* digestion (Seiquer *et al.*, 2015), while others denoted a significant decrease in the post-digestion TPC (Campos-Vega *et al.*, 2015) which implies that the impact of this process differ according to the nature of the food, its bioactive composition and its concentration.

The increase of TPC amount and overall antioxidant activity could be explained by the release of phenolic compounds and other bioactive molecules after undergoing the simulated digestion. In fact, some phenolic compounds bound to proteins tend to undergo hydrolysis and to be liberated under the effect of digestive enzymes and pH conditions and therefore can be extracted more efficiently (Hachibamba *et al.*, 2013). Moreover, depending on their chemical structure, polyphenols are affected by the intestinal enzyme action by a different way, leading to derivatives which could strongly affect their biological and antioxidant activity (Campos-Vega *et al.*, 2015). Although the accurate reasons behind the different kind of changes affecting the antioxidants amount and activity are still unknown, the enzymes-pH-

macromolecules interactions that happen during the digestion phases are claimed to be the main responsible factor (Baker *et al.*, 2013). In addition, *in vitro* digestion studies performed in saffron infusions suggested that polyphenols may enhance the bioaccessibility of other antioxidant compounds, protecting them during digestion due to their radical scavenging properties (Ordoudi *et al.*, 2015).

The residual fractions obtained after the *in vitro* digestion of foods are considered theoretically inaccessible parts that cannot cross the small intestine and hence they are usually disregarded in the bioavailability studies. Most of the non-released compounds are fermented or discharged in the feces (Chandrasekara & Shahidi 2011). However, a small part of the residues could undergo a surface reaction and exert a local antioxidant activity or later be subjected to microbial degradation leading to the release of a wide variety of biomolecules (Denev *et al.*, 2012). In an attempt to investigate the presence of any phenolic content and a possible local antioxidant activity in the residues, the residual fractions R1, R2 and R3 obtained respectively from I1, I2 and I3 after *in vitro* digestion were evaluated. Data shown in Table 3 indicate that the residual fractions still contained polyphenolic compounds and antioxidant activity. In a similar manner to that observed for infusions and bioaccessible fractions, the initial concentration of the plant significantly affected the residual TPC and antioxidant properties (DPPH, ABTS and FRAP) after *in vitro* digestion, that varied as follow: $R1 > R2 > R3$.

These results revealed that a part of the compounds left in the inaccessible part are phenolic compounds and/or have antioxidant potential. On average, 17 % of the phenolic compounds found after the digestive process remain in the residual fraction (Figure 1). In addition, 32% of the DPPH activity, 8% of ABTS and 12% of the reducing power, were located as non-bioaccessible. In fact, some large polyphenols like proanthocyanidins or those that form

interactive associations with proteins are inaccessible to gastrointestinal enzymes and therefore cannot be digested (Gleichenhagen & Schieber 2016). Although it is hard to predict the exact pathway of the residual antioxidants, an antioxidant local activity is very probable especially since the intestine is vulnerable to free radicals and needs various defense mechanisms (Halliwell *et al.*, 2000).

Cell culture assays

The bioaccessible fraction of sample 1, with the highest yield of TPC and antioxidant potential, was selected for the subsequent experiments in Caco-2 cells. Dilution 1:2 (B1:DMEM) was used, since preliminary viability assays showed that at such conditions cell viability was never <85%.

Absorption across Caco-2 monolayers assay

Results of absorption of phenolic compounds and antioxidant activity across Caco-2 cell monolayers are summarized in Table 4. After 2h of incubation with the digested infusion of *C. ipecacuanha*, the 75% from the initial TPC exposed to cells were absorbed. In addition, a significant proportion of the anti-radical activity (80% and 50% for DPPH and ABTS, respectively) and ferric reducing power (65%) was recovered in the basal chambers.

As aforementioned, there are no previously existing data about the bioavailable polyphenols content in neither *C. ipecacuanha* extract nor infusion. The current study shows for the first time that antioxidant compounds from the digested infusions of *C. ipecacuanha* present a good rate of absorption through intestinal cells, and that antioxidant activity is maintained after absorption.

In this test, Caco-2 was used as a model to assess the bioavailability of phenolic compounds. It has been reported that using this method to mimic the intestinal absorption is quite efficient

since Caco-2 cells express the typical morphological and functional properties as well as the enzymatic activities of small bowel enterocytes (Lea, 2015). According to literature, Caco-2 cell line model has been extensively used to study the bioavailability of a large number of nutrients and bioactive compounds with antioxidant properties, such as polyphenols and terpenoids (Pérez-Sánchez *et al.*, 2017; Achour *et al.*, 2018) or carotenoids (Liu *et al.*, 2004). Furthermore, a high correlation between the Caco-2 permeability data and the human enterocytes absorption coefficients *in vivo* was demonstrated (Lea 2015, Chopra *et al.*, 2010).

However, this system may have some limitations in determining precisely the absorption rate as it happens inside the human body. In fact, it has been suggested that the apical to basolateral transport of compounds across the Caco-2 monolayer is up to 100 fold slower than the transport in the small intestine (Artursson *et al.*, 2001). In addition, the *in vivo* physiological conditions could not be accurately replicated *in vitro* since there is no blood capillary network in experimental conditions. Another important factor is the complexity of the absorption phenomenon. Although polyphenols are one of the most common micronutrients in our diet, they are not necessarily the most available or active. Polyphenols have been considered poorly absorbed by the small intestine, highly metabolized or rapidly eliminated (Rein *et al.*, 2013). However, some of them, like hydroxytyrosol and tyrosol, have been demonstrated to be highly bioavailable (Miro-Casas *et al.*, 2003). In this sense, the rate and extent of intestinal absorption of polyphenols is determined by their specific chemical structure, as in their native form polyphenols cannot be absorbed (with the exception of anthocyanins) and must be hydrolyzed by the intestinal enzymes (D' Archivio *et al.*, 2010). Studies with Caco-2 cells, under conditions closely resembling the human digestive tract, have found that the absorption of flavonoid and isoflavone aglycones form was much more efficient than the glycoside ones (Liu & Hu, 2001; Murota *et al.*, 2004). Recently, Achour *et*

al. (2018) have observed that certain polyphenols from rosemary infusion show improved bioavailability in Caco-2 cells compared to pure compounds, and that polyphenols undergo metabolic transformation during incubation with cultured intestinal cells. In addition, other factors like food processing, interactions with other components of the food matrix and synergistic effects could have a major effect on polyphenols bioavailability (D'Archivio *et al.*, 2010). Besides, before intestinal absorption, polyphenols naturally existing as esters, glycosides and polymers will have to face enzymatic hydrolysis and microflora activity, which, together with the polyphenols diversity (molecular weight, structure and amount) play an important role in determining the absorption phenomenon (Pandey & Rizvi 2009, Manach *et al.*, 2004).

Reactive oxygen species (ROS) assay

The health-promoting effect of plants antioxidants is thought to arise from their protective effects by counteracting ROS (Hlel *et al.*, 2017). One of the most important reasons for encouraging the consumption of polyphenol rich foods is their antioxidant activity such as scavenging free radicals, inhibiting ROS and preventing oxidative stress related diseases (Ahmed *et al.*, 2011; Ghali *et al.*, 2013). Thus, it is not enough for polyphenols to be bioaccessible for absorption but also to exert beneficial activities, namely preventing ROS generation that could lead to permeability changes of the intestine, ulcerative colitis, inflammatory bowel syndromes and a variety of intestinal diseases (Grisham 1994). It is at utmost importance to check the ability of the samples to inhibit ROS production in the intestine at a cellular level. Therefore, we assessed the ROS inhibition activity in Caco-2 cells by *C. ipecacuanha* digested infusion in order to evaluate its efficacy. In the first part of experiment, we tested the basal effect via monitoring the fluorescence level and comparing it to the control. Under basal conditions, there was a strong decrease of ROS generation after 50

min when the cells were treated with B1 sample comparing to the DMEM, used as control (Figure 2); even after 90 min of incubation the fluorescence continue to decline as the effect of the sample increases.

In a second attempt to further evaluate the sample's activity, we induced oxidation using t-BOOH as a pro-oxidant agent, which led to a strong increase in the ROS production. When cells were pre-incubated with B1 the ROS generation was remarkably neutralized compared with control cells, after 50 min and 90 min of treatment (Figure 2) which reveals that the liberated post digested polyphenols possess the ability to inhibit and counteract free radicals, thus protecting cells from oxidation. Experiments conducted with the post-digestion bioaccessible fraction from polyphenol-rich argan and extra virgin olive oil in Caco-2 cells have reported a similar protective effect against induced oxidation (Seiquer *et al.*, 2015).

The lack of information regarding the phenolic compounds of *C. ipecacuanha* makes it hard to propose the compounds responsible for the antiradical activity. Nevertheless, it is well established that polyphenols could work as single compounds, in a synergy or in a complementary way to boost the overall intracellular antioxidant activity. It has already been demonstrated that gallic acid and syringic acid, two phenolic acids, can protect Caco-2 cells against oxidative damage by enhancing glutathione reductase and peroxidase activities, (Wang *et al.*, 2017). Moreover, other components present in the *C. ipecacuanha* root, such as alkaloids or cyclotides (Fahradpour *et al.*, 2017, Nomura & Kutchan 2010) could contribute to the antioxidant properties found in the present study. These findings suggest a possible biological relevance associated with the traditional use of ipecac preparations.

Conclusions

In this paper, the total phenolic content and antioxidant properties of *C. ipecacuanha* infusions before and after an *in vitro* digestion process was investigated under different initial concentrations of the root sample. It was found that a water volume of 50 mL per gram of sample provided the maximum yield of extraction of TPC and antioxidant activity. The *in vitro* digestion promoted an increase of releasing phenolics and free radical scavenging properties, which were detected both in bioaccessible and residual fractions after digestion.

Using Caco-2 cell culture models, it was shown that antioxidant compounds (mainly polyphenols) from bioaccessible fractions were well absorbed across intestinal monolayers and were able to reduce ROS generation against an induced oxidative stress.

From the outcome of this investigation, it is possible to conclude that ipecac infusion polyphenols are highly bioavailable and effective at the cellular level. We suggest considering *ipecac* as a promising functional food ingredient provided further investigation are required to be carried out to determine the safety and the right dose of consumption.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1

Color parameters of *C. ipecacuanha* infusions obtained by the CIELAB method (L^* , a^* , b^*).

Sample	L^*	a^*	b^*
I1	49.51 ± 0.00^c	-0.98 ± 0.01^c	9.53 ± 0.01^c
I2	47.20 ± 0.01^b	-0.81 ± 0.00^b	7.16 ± 0.01^b
I3	44.54 ± 0.05^a	-0.72 ± 0.02^a	4.71 ± 0.03^a

Data are means $\pm$ SD (n=3). Values within a column with different superscripts are significantly different ($P < 0.05$). I1: 0.5g/25ml, I2: 1g/25ml and I3: 3g/25ml.

Table 2

Total phenolic content (TPC) and antioxidant activity (DPPH, ABTS and FRAP) determined in *C. ipecacuanha* infusions (I1, I2, I3) and bioaccessible fractions (B1, B2, B3) obtained after *in vitro* digestion.

Sample	TPC (mg GA/g plant)	DPPH (mmolTrolox/g)	ABTS (μ mol Trolox/g)	FRAP (μ mol Trolox/g)
I1	91.01 $\pm$ 4.46 ^c	2.39 $\pm$ 0.15 ^c	80.51 $\pm$ 0.10 ^c	18.33 $\pm$ 2.1 ^c
I2	79.92 $\pm$ 1.61 ^b	0.94 $\pm$ 0.01 ^b	39.24 $\pm$ 0.54 ^b	13.34 $\pm$ 0.70 ^b
I3	15.37 $\pm$ 0.14 ^a	0.19 $\pm$ 0.06 ^a	13.40 $\pm$ 0.18 ^a	11.05 $\pm$ 1.6 ^a
B1	126.54 $\pm$ 4.31 ^c	3.36 $\pm$ 0.06 ^c	101.44 $\pm$ 0.21 ^c	17.78 $\pm$ 0.76 ^c
Increase (fold)	1.39	1.40	1.26	0.97
B2	87.38 $\pm$ 2.65 ^b	2.02 $\pm$ 0.03 ^b	54.44 $\pm$ 0.77 ^b	12.11 $\pm$ 0.98 ^b
Increase (fold)	1.09	2.15	1.39	0.91
B3	31.93 $\pm$ 1.22 ^a	0.42 $\pm$ 0.03 ^a	18.35 $\pm$ 0.13 ^a	7.80 $\pm$ 0.82 ^a
Increase (fold)	2.08	2.21	1.37	0.71

Data are means $\pm$ SD (n=3). Values within a column in each section with different superscripts are significantly different. I1: 0.5g/25ml, I2: 1g/25ml and I3: 3g/25ml. B1: bioaccessible fraction from I1 infusion B1: bioaccessible fraction from I2 infusion B3: bioaccessible fraction from I3 infusion

Table 3

Total phenolic content (TPC) and antioxidant activity (DPPH, ABTS and FRAP) determined in residual fractions (R1, R2, R3) obtained after *in vitro* digestion of *C. ipecacuanha* infusions.

	TPC	DPPH	ABTS	FRAP
Sample	(mg GA/g plant)	(mmolTrolox/g)	(μ mol Trolox/g)	(μ mol Trolox/g)
R1	9.65 ± 1.33^c	0.94 ± 0.05^c	4.93 ± 0.11^c	2.31 ± 0.53^c
R2	3.12 ± 0.31^b	0.78 ± 0.04^b	3.84 ± 0.10^b	1.98 ± 0.26^b
R3	1.38 ± 0.57^a	0.35 ± 0.00^a	2.79 ± 0.15^a	1.02 ± 0.33^a

Data are means $\pm$ SD (n=3). Values within a column in each section with different superscripts in each are significantly different. R1: bioaccessible fraction from I1 infusion, R2: Residual fraction from I2 infusion R3: Residual fraction from I3 infusion

Table 4

Total initial content in apical chambers, recovered content in basal chambers and absorption of TPC and antioxidant activity (DPPH, ABTS and FRAP) in Caco-2 cell assays after 2 h incubation with bioaccessible fractions of *C. ipecacuanha* infusion.

	Initial content (mg/well)	Recovered content (mg/well)	Absorption (%)
TPC	3.61 ± 0.42	2.73 ± 0.24	75.52
DPPH	1.27 ± 0.01	1.02 ± 0.12	80.32
ABTS	0.35 ± 0.00	0.18 ± 0.07	52.14
FRAP	0.09 ± 0.01	0.06 ± 0.01	64.83

Data are means ± SD (n=5). Absorption was calculated as the percentage transported across the cell monolayer from the initially loaded in the apical chamber.

Figure 1. Contribution of TPC and antioxidant activity (DPPH, ABTS and FRAP) from bioaccessible and residual fractions to the total recovered after *in vitro* digestion of *C. ipecacuanha* infusions, expressed as percentage.

Figure 2. ROS generation (expressed as units of fluorescence) in Caco-2 cells after incubation with bioaccessible fractions (B1) of *C. ipecacuanha* infusions (basal effect) and in cells pre-treated with B1 and oxidized with 5 mM t-BOOH (protective effect). Control: cells treated with culture medium. Values are means $\pm$ SD (n=5).

Supplementary material

Figure S1. The general plan followed in studying *Carapichea ipecacuanha* infusions.