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Original Article

Inhibition of HMG-CoA reductase activity and cholesterol permeation through Caco-2 cells by caffeoylquinic acids from *Vernonia condensata* leaves

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ABSTRACT

The aim of this study was to provide scientific knowledge to support the use of *Vernonia condensata* Baker, Asteraceae, beverages for their alleged hypocholesterolemic properties by testing their action as HMG-CoA reductase inhibitors and their capacity to lower dietary cholesterol permeation. Chlorogenic acid, and other caffeoylquinic acids derivatives were identified as the main components of these beverages by LC–MS/MS. No changes in the composition were notice after the *in vitro* gastrointestinal digestion and no toxicity against Caco-2 and HepG2 cell lines was detected. Cholesterol permeation through Caco-2 monolayers was reduced in 37% in the presence of these herbal teas, and the caffeoylquinic acids permeated the monolayers in 30–40% of their initial amount in 6 h. HMG-CoA reductase activity was reduced with these beverages, showing an IC_{50} of 217 $\mu g\, ml^{-1}$. It was concluded that caffeoylquinic acids, the major components, justified 98% of the enzyme inhibition measured.

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Introduction

The reduction of blood cholesterol level is one of the guidelines of the European Society of Cardiology to reduce the risk of developing cardiovascular diseases (Backer et al., 2003), one of the highest cause of death in Europe and also around the world (Nichols et al., 2014). Atherosclerosis is a disease in which atheromatous plaques are formed inside arteries, triggering their hardening and narrowing, limiting, the blood flow. These plaques are formed essentially by triacylglycerols, lipoproteins, cholesterol and foam cells (Niki, 2011; Bentzon et al., 2014). Reduction of atherosclerosis by diminishing the blood cholesterol level is one of the approaches for decreasing the risk of heart diseases (Gotto and Phill, 2011). This may be achieved by decreasing diet cholesterol intake, as some studies reported correlations between diet cholesterol intake and blood cholesterol level (Houston et al., 2011; Dehghan et al., 2012), which can contribute for atherosclerosis-related diseases (Niki, 2011). Besides a healthy diet (Reiner et al., 2011) cholesterol levels can be decreased by reducing its biosynthesis through inhibition of the enzyme HMG-CoA reductase (HMGR), involved in the cholesterol biosynthetic pathway (Istvan and Deisenhofer, 2001), which is the approach taken clinically by statins. Another approach is the reduction in dietary cholesterol absorption through the blockage of the cholesterol protein transporter (Niemann-Pick C1-like 1 protein – NPC1L1), in intestinal cells (Wang, 2007), which can be accomplished by the drug ezetimibe (Vrablik et al., 2014). Hypercholesterolemia can even be treated simultaneously by ezetimibe and statins, one to reduce cholesterol absorption and the other to inhibit its synthesis (Hamilton-Craig et al., 2010).

Physical activity with other lifestyle corrections, such as a nutritionally balanced diet rich in fruits and vegetables, are naturally effective factors for preventing the risk of cardiovascular diseases (Rodriguez-Mateos et al., 2014). In fact, the effects of different polyphenol-containing foods and beverages on health have been analyzed in several studies that showed that fruit, vegetables, nuts and plant-derived beverages, such juices and infusions/decoctions, with a high antioxidant activity may play an important role preventing various disorders associated with oxidative stress, such as

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cancer, cardiovascular and neurodegenerative diseases (Scalbert et al., 2005; Wolfe et al., 2008). Evidence has been accumulated in recent years on the diversity of bioactivities of components of herbal decoctions or infusions, such as phenolic compounds (Al Shukor et al., 2013), namely the hypocholesterolaemic effect (Chen et al., 2008; Johari et al., 2015). *Vernonia condensata* Baker, Asteraceae, commonly named in Brazil as "boldo-baiano", "alumã" or "figatil", is a shrub probably native from Tropical Africa and taken to Brazil in colonial times (Toyang and Verpoorte, 2013). *Vernonia condensata* is a species frequently referred by a number of synonyms such as *V. amygdalina* Delile, *V. bahiensis* Toledo, *V. sylvestris* Glaz. and after Robinson's taxonomical revision of the paleotropic species of tribe Vernonieae (1999) as *Vernonanthura condensata* (Baker) H. Rob. However Robinson's classification of the New World Vernonieae species, formerly named as *Vernonia* sensu Baker, into new genera has not generally been accepted (De Oliveira et al., 2007). Authors consider that the taxonomical classification of the genus *Vernonia* Schreb is complex, yet unclear and needing further studies (Martucci et al., 2014). In Brazil, leaves of *V. condensata* are often used to prepare beverages in the form of juices or infusions/decoctions. Despite the numerous phytochemical and pharmacological studies carried out in species of *Vernonia* genus (Toyang and Verpoorte, 2013), only a few scientific articles have been published concerning *V. condensata*, although recently it has been reported that its ethanol extracts can be a source of bioactive compounds with antioxidant activity (Da Silva et al., 2013). There are also reports on the activity of this herb as hypolipidemic and hypocholesterolemic (Pizziolo et al., 2011). The current study aimed to evaluate the composition and the biological activity of beverages prepared from *V. condensata* leaves infusions and decoctions in cholesterol-lowering permeation through the intestinal barrier using Caco-2 cell monolayers, together with the inhibition of HMGR activity, in an attempt to contribute with a scientific explanation for the effect of these beverages on human health.

Materials and methods

Plant material

Leaves of *Vernonia condensata* Baker, Asteraceae, Vernonieae, were collected from plants cultivated in the Garden of Medicinal Plants of Universidade Estadual de Santa Cruz (Bahia, Brazil) between September and December of 2012. A voucher specimen of this species was deposited in the Herbarium of this University (HUESC 16275).

Beverages preparation

Vernonia condensata leaves were prepared as infusions and decoctions, using 40 g of dry leaves in 400 ml of distilled water. For infusions the plant material was immersed in freshly boiled water during 10 min and for decoctions the material was boiled in water for 10 min. After cooling infusions and decoctions were filtered through a grade 1 Whatman paper and lyophilized to obtain dried mixtures. The yield of dry material was 2.5% in infusions and 3% in decoctions.

Chemical analysis by HPLC-DAD and LC–MS

The analysis of the infusion and decoction was carried out by HPLC-DAD and the identification and quantification of their chemical components, was accomplished using standards chlorogenic acid (Sigma–Aldrich, Spain), cynarin (Sigma–Aldrich, Spain), luteolin and kampherol obtained from Sigma–Aldrich, Spain. Both the extract and the standards were analyzed using an Elite LaChrom® VWR Hitachi liquid chromatograph equipped with a Column Oven L-2300 and Diode Array Detector L-2455 (VWR, USA) (Falé et al., 2013c). Standards of cynarin and chlorogenic acid, the main components, were run under the same conditions using 0.1 mg ml^{-1} solutions in methanol (Merck, Darmstdt, Germany). The extracts were analyzed by HPLC injecting 25 μl (1 mg ml^{-1}) with an auto injector, and using a gradient composed of solution A (0.05% trifluoroacetic acid), and solution B (methanol) as follows: 0 min, 80% A, 20% B; 20 min 20% A, 80% B; 25 min, 20% A, 80% B. the flow was 1 ml min^{-1} and the detection was carried out between 200 and 500 nm with a diode array detector. The column used was a LiChroCART® 250-4LiChrospher® 100 RP-8 (5 μm) column (Merck, Darmstadt, Germany).

The LC–MS and LC–MS/MS analysis were carried out on a liquid chromatograph Surveyor Plus Modular LC system connected to a LCQ Duo ion trap mass spectrometer equipped with an electrospray ionization (ESI) source, from Thermo Scientific (Bremen, Germany) (Falé et al., 2013c). The extracts were analyzed by LC–MS/MS injecting 25 μl with a concentration of 10 mg ml^{-1} and using a linear gradient composed of solution A (1.0% formic acid), and solution B (methanol) as follows: 0 min, 70% A, 30% B; 20 min 20% A, 80% B; 25 min, 20% A, 80% B. The mass spectrometer was operated in both positive and negative ion modes in the range 120–1000 m/z, and the parameters were adjusted in order to optimize the signal-to-noise ratios (S/N) for the ions of interest. Briefly, the nebulizing and auxiliary gas (nitrogen) flow rates were 40 and 20 (arbitrary units) and the capillary temperature was set to 250 °C. Collision induced dissociation (CID) experiments were performed by isolating the ions within the ion trap and accelerating them in order to suffer multiple collisions, with the background gas present in the ion trap (helium) using a data dependent acquisition mode. The ions of interest were activated by applying a percentage of a supplementary a.c. potential in the range of 0.75–1.75 Vp-p (peak-to-peak) to the end cap electrodes of the ion trap at the resonance frequency of the selected ion (referred to as the normalized collision energy, NCE). The injection times were 50 ms in a full scan and 200 ms in an MS/MS scan. Xcalibur™ software from thermo scientific was used to acquire and process the data.

Toxicity tests

The toxicity of the *V. condensata* decoctions was analyzed by thiazoyl tetrazolium bromide (MTT) (Sigma–Aldrich, Barcelona, Spain) method using Caco-2 (ATCC#HTB37) and HepG2 (ATCC#HB-8065) cell lines, according to Mosman method (Mosman, 1983). Briefly: Caco-2 and HepG2 were cultured in DMEM supplemented with 10% FBS, 100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin, and 2 mM L-glutamine at 37 °C in an atmosphere with 5% CO_2. The medium was changed every 48–72 h, and the cells were passaged before reaching confluence. Cytotoxicity studies were performed in 96-well plates, exposing the cells for 24 h to several concentrations of the plant extracts in culture medium. The average and standard deviation were calculated from 3 × 8 replicates for each concentration.

In vitro *digestion with gastric and pancreatic juices*

The *in vitro* metabolism of the *V. condensata* decoction was studied by the method described in Porfirio et al. (2010).

Biological activities evaluation of Vernonia condensata *decoction*

The antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma–Aldrich, Barcelona, Spain) method, as described previously (Porfirio et al., 2010). Inhibition of HMG-CoA reductase was studied using a kit provided by Sigma (Barcelona, Spain). The assay was performed as described by Falé

et al. (2013c) by quantifying NADPH at 0, 1, 2, 4 and 6 min in reaction mixtures containing different concentrations of *V. condensata* decoctions. The percentage of HMGR inhibition by each decoction concentration was calculated as: $I\,(\%) = 100 - 100 \times (\upsilon_{sample}/\upsilon_{control})$, where I is the percentage inhibition of enzyme, υ_{sample} is the initial rate of enzymatic reaction for the decoction and $\upsilon_{control}$ is the initial rate for the control reaction in the absence of extract. The IC_{50} value was calculated and represents the concentration of the decoction that inhibited 50% of HMGR activity. Simvastatin, a commercially available drug to lower cholesterol, was purchased from Sanofi-Aventis (Porto Salvo, Portugal) and used as a positive control.

Permeation studies

For the permeation of the beverage components and cholesterol, Caco-2 cells, previously grown as described in Falé et al. (2014) were seeded in 12-well transwell plate inserts at a density of 2–4 × 10^4 cells/cm^2. These inserts had 10.5 mm diameter and 0.4 μm pore size (BD Falcon™). The monolayers were formed till confluence was attained and then differentiated (21–26 days). The medium was replaced every 24 h. The membranes were adequate to permeability studies when the transepithelial electrical resistance (TEER) was higher than 250 Ω cm^2. This value was measured with a Millicell ERS-2 V-Ohm Meter. To start the assay of the permeation of the decoction components, the cells grown in the transwells plate inserts were washed with HBSS (VWR International, Lisbon, Portugal) and then 0.5 ml of the decoction in HBSS (0.5 mg of dried mixture ml^{-1}) were applied into the apical chamber. In the basolateral chamber 1.5 ml of HBSS were applied. The cells were in contact with the decoction during 6 h at 37 °C, 5% CO_2. The solutions from both chambers were collected and 25 μl aliquots were analyzed by HPLC-DAD as described. The permeation of the decoction components was quantified in percentage of component detected in the basolateral chamber relatively to quantity introduced in the apical chamber per hour per Caco-2 cell monolayer area (cm^2).

For the study of the effect of the *V. condensata* decoction in cholesterol permeation, the Caco-2 cells grown in transwell plate inserts were washed with HBSS and 0.5 ml of solution containing decoction (0.5 mg dried mixture ml^{-1}) plus cholesterol (2 mg ml^{-1}) in HBSS was added to the apical chamber. In the basolateral chamber 1.5 ml of HBSS were applied. The cells were in contact with this solution during 6 h at 37 °C, 5% CO_2. After this period the solutions from both chambers were collected and analyzed by HPLC-DAD. 25 μl of each chamber were injected in the HPLC-DAD system described, however the separation was carried in isocratic mode using 50% methanol plus acetonitrile for 15 min with a flow of 1 ml min^{-1}. Detection of cholesterol was carried out at 210 nm. The effect of ezetimibe (bought from MSD-SP Limited, Hertfordshire, UK), a commercial drug to lower cholesterol, was analyzed by introducing 100 mM dissolved in the culture medium in the apical chamber and allowed to be into contact with the cells, in the presence of cholesterol, in the same concentration as used in the control. The permeation was quantified in percentage of cholesterol area detected in the basolateral chamber and inside the cells relatively to quantity introduced in the apical chamber, measured as area in the HPLC-DAD system, converted into molarity (nmol) per hour per Caco-2 cell monolayer area (cm^2). The cells were washed with HBSS, scraped and resuspended in HBSS. The cells were sonicated 5 × 10 s, centrifuged 10 min at 5000 × *g*, and the supernatant was analyzed by HPLC-DAD using the same method as described before.

For the study of the effect of the 24 h incubation of cells with *V. condensata* decoction in the cholesterol permeation, the Caco-2 cells grown in transwell plate inserts were in contact with 0.5 ml of the decoction in DMEM medium (0.5 mg dried mixture/ml), during 24 h at 37 °C, 5% CO_2. After this period the cells were washed with HBSS and cholesterol was added to the apical chamber (2 mg/ml HBSS). In the basolateral chamber 1.5 ml of HBSS were applied. The cells were in contact with the cholesterol during 6 h and the solutions from both chambers were collected and analyzed by HPLC-DAD using the isocratic mode. The same approach and conditions were used to study the effect of ezetimibe in cholesterol permeation. Triplicate assays were performed for each analysis.

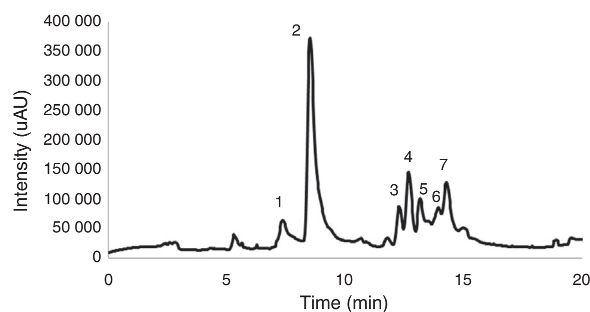

Fig. 1. HPLC-DAD from decoctions of *Vernonia condensata* leaves. Peak number refers to Table 1.

Data analysis

All data analysis was performed using Microsoft Excel 2010 and the results were expressed as means ± standard deviation. Additional analysis of variance (ANOVA) was performed at a confidence level of $p = 0.05$ (Lison, 1968).

Results and discussion

Components of the herbal beverages

The beverages prepared by infusion and decoction of *Vernonia condensata* leaves were analyzed by HPLC-DAD and their phenolic compounds identified by LC–MS/MS. As the chromatograms of infusions and decoctions were similar, having decoctions only a higher quantity of all the chemical components, the study proceeded using only this beverage. The chromatogram obtained is shown in Fig. 1 and the identified compounds are included in Table 1.

As it can be seen in the HPLC chromatogram, most of the constituents are caffeic acid derivatives (peaks 1–5, 7), only compound number 6 was identified as a flavonoid derivative.

The presence of chlorogenic acid and cynarin in the decoction was confirmed by HPLC-DAD and mass spectrometry using the respective standards. Chlorogenic acid (3-caffeoylquinic acid, peak number 2) is the main component of the decoction, followed by dicaffeoylquinic acids. Mono and di-esters of caffeic acid with quinic acid have been also detected in several Vernonia species (as reviewed by Toyang and Verpoorte, 2013) and chlorogenic acid is considered a biomarker of Asteraceae (Jaiswal et al., 2011). In other species of this family, such as *Cynara cardunculus*, caffeoylquinic acids are the infusion's main components, and among them chlorogenic acid and cynarin were also detected (Falé et al., 2013c).

Cytotoxicity, antioxidant activity and digestibility of the leaves V. condensata *decoction*

Before initiating the permeation studies with the decoction through the intestinal barrier, and the detection of the HMGR activity, the cytotoxicity of the herbal tea toward HepG2 and Caco-2 cells lines was evaluated. The decoction of *V. condensata* leaves showed an IC_{50} of 0.58 mg ml^{-1} either in HepG2 or Caco-2 cells (Table 2). The IC_{50} values obtained with the two cell lines tested were higher than

Table 1
Major constituents of decoctions from *Vernonia condensata* leaves. Identification by LC–MS/MS, in negative ion mode and relative abundance of each compound (%) found in the HPLC-DAD chromatogram.

Peak number	HPLC peak Retention time	$[M-H]^{-1}$ (m/z)	Fragment ions (m/z) (Rel. Ab. %) MS2	Compound	Compound (%)	Quantity (μg/mg)
1	7.5	353	259 (11), 179 (62), 173 (100), 135 (11)	Caffeoylquinic acid	5	1.2[a]
2	8.6	353	192 (10), 191 (100), 179 (12),	Chlorogenic acid	34	7.8
3	12.4	515	353 (100), 335 (11), 299 (5), 255 (5), 203 (6), 191 (5), 179 (9), 173 (12)	Dicaffeoylquinic acid	8	2.7[b]
4	12.9	515	353 (100), 203 (1), 191 (3), 179 (2), 173 (1)	Dicaffeoylquinic acid	12	4.5[b]
5	13.4	515	353 (100), 335 (3), 191 (9), 179 (1)	Cynarin	9	3.2
6	14.0	461	381 (5), 369 (2), 357 (8) 355 (2), 327 (4), 285 (100)	Luteolin	7	–
7	14.5	515	353 (100), 335 (2), 317 (6), 299 (21), 255 (8), 204 (3), 203 (17), 179 (3), 173 (7)	Dicaffeoylquinic acid	11	4.0[b]

[a] Equivalent of chlorogenic acid.
[b] Equivalents of cynarin.

the established limit of IC_{50} value (0.1 mg ml^{-1}), that is considered usually as toxic to human cell lines (Oonsivilai et al., 2007).

These results are in agreement with those previously found *in vivo* studies, which demonstrated that the tea from leaves of V. condensata has no oral acute toxicity in mice (Monteiro et al., 2001). Aqueous extracts of other Asteraceae species, such as *Cynara cardunculus*, with similar composition to *V. condensata* decoctions, also did not shown any toxicity to Caco-2 and HeLa cell lines (Falé et al., 2013c).

As no cytotoxicity was detected in the decoction of *V. condensata* leaves, the effect of the *in vitro* digestive process in this beverage could be evaluated. So, the decoction was subjected to artificial gastric and pancreatic juices simulating the conditions in the stomach and small intestine, respectively. The changes in the chemical composition of the decoction were followed by analyzing the digested extract hourly by HPLC. As it can be observed in Fig. 2, even after 4 h of digestion with gastric and pancreatic juices, the composition remained similar to the initial beverage. This suggests that the decoction components are stable under the gastric and pancreatic pH conditions and are not substrates of the enzymes in the gastrointestinal tract. These findings are in agreement with others reported for this class of compounds (Bouayed et al., 2012; Falé et al., 2013c, 2014).

The antioxidant activity, quantified by the free radical DPPH method, revealed that 20.0 ± 0.6 μg ml^{-1} of the decoction of *V. condensata* leaves had the capacity to extinguish 50% of the compound absorption (Table 2). The EC_{50} value for the antioxidant activity of *V. condensata* decoction was in the same magnitude of that obtained for the commercial antioxidant BHT (15.7 ± 0.2 μg ml^{-1}) (Mata et al., 2007). Previous studies concerning the detection of the *in vitro* antioxidant activity in ethanol extracts and fractions obtained from *V. condensata* leaves have shown that this species has potential to be an important source of antioxidant compounds (Da Silva et al., 2013). The antioxidant activity studied here confirms this conclusion and suggests that the decoction of *V. condensata* leaves has a good capacity to capture electrons inside the cell.

Table 2
Decoction of *Vernonia condensata* leaves – toxicity in HepG2 and Caco-2 cells lines (IC_{50} μg ml^{-1}) *in vitro* digestion and antioxidant activity (EC_{50} μg ml^{-1}).

Toxicity	
HepG2 cells	$IC_{50} = 586.3 \pm 6.5$
Caco-2 cells	$IC_{50} = 580.0 \pm 10.0$
Digestion[a]	
Pepsin	No modification
Pancreatin	No modification
Antioxidant activity[b]	
V. condensata	$EC_{50} = 20.0 \pm 0.6$
BHT	$EC_{50} = 15.7 \pm 0.2$[c]

[a] Digestion carried out using pepsin at pH 1.2 during 4 h and pancreatin at pH 8.0 during 4 h (Porfirio et al., 2010).
[b] Antioxidant activity was measured using DPPH solution incubated for 30 min in the presence of different extract concentration and the absorbance was read at 517 nm against a blank (Porfirio et al., 2010).
[c] Mata et al. (2007).

Effect of the decoction in cholesterol permeation through Caco-2 cells

Caco-2 monolayers can be used to study the permeation through the intestinal barrier of compounds taken orally (from diet or drugs), as these cells can differentiate on polycarbonate membranes, acquiring a phenotype with tight junctions, microvilli and a number of enzymes and transporters characteristic of enterocytes

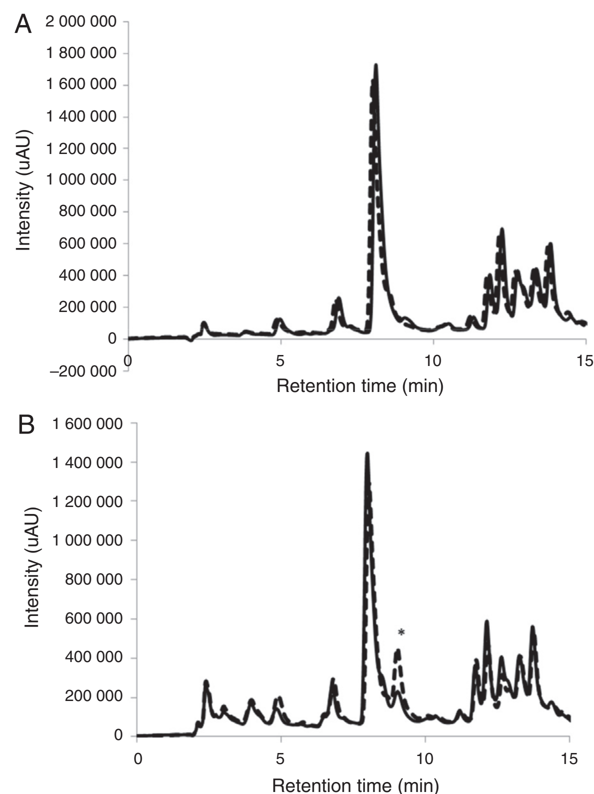

Fig. 2. HPLC chromatograms of the decoction of *Vernonia condensata* leaves before and after digestion with artificial gastric and pancreatic juices (initial time; ---- after 4-h incubation with artificial juices): (A) *in vitro* gastric digestion; (B) *in vitro* pancreatic digestion. *Indicates the pancreatin peak.

(Hidalgo et al., 1989). This system has been recognized by FDA as appropriate to study permeation of drugs (Hu et al., 2004). As the decoction of *V. condensata* leaves was not toxic to Caco-2 cells and no changes in the chemical composition were noticed after the *in vitro* digestion, the permeation studies were initiated by introducing 0.5 mg ml^{-1} of decoction in the apical cell culture medium, and allowed to be into contact with the cells for 6 and 24 h. The control had only cholesterol at a concentration of 5 mM and the test wells contained cholesterol together with the *V. condensata* decoction at a concentration of 0.5 mg ml^{-1}. Cholesterol was measured inside the cells and in the baso-lateral compartment. Ezetimibe, the drug used to reduce cholesterol absorption from the diet, was also tested as a positive control at a concentration of 100 μM (Feng et al., 2010). The permeation of cholesterol to the basolateral compartment of Caco-2 cells, when the extract was into contact with these cells for 6 h, was not modified relatively to the control. Infusions from other Asteraceae species containing mainly chlorogenic acid and dicaffeoylquinic acids, like those of *Cynara cardunculus*, did not inhibit the transport of cholesterol through Caco-2 cell monolayers when the test was carried out for 6 h (Falé et al., 2014). When the study was prolonged for 24 h, that is when the extract was into contact with the cells for 24 h and cholesterol was then applied and quantified afterwards, a lowering tendency could be noticed on the cholesterol level on the basolateral compartment of the cells, although not statistically significant at 95% confidence level ($p = 0.05$). When analyzing the intracellular value of cholesterol, it can be seen that the *V. condensata* decoction reduced its level, being the differences statistically significant ($p = 0.05$). Ezetimibe inhibited both the permeation of cholesterol to the basolateral compartment and to inside the cell. The reduction in the permeation of cholesterol can be associated with the down-regulation of mRNA Niemann-Pick Like 1 protein (NPC1L1) that promotes the transport of cholesterol inside the cells as well as with other membrane transporter proteins all involved either in the uptake or with the efflux of cholesterol from the cell (Liang et al., 2015).

Permeation of phenolic compounds present in the tea and inhibition of HMRG activity

To have any effect on the enzyme activity inside the cells it is necessary that the active phenolic compounds present in *V. condensata* decoction permeate the intestinal barrier. In the current study the decoction was introduced in the culture medium for 24 h at a concentration of 0.5 mg ml^{-1}. In the beginning and at the end of the experiment, aliquots were withdrawn and analyzed by HPLC-DAD. The compounds detected in the basolateral compartment were chlorogenic acid and cynarin. The permeation values through the intestinal barrier simulated by Caco-2 cells were 26% and 15.8%, for chlorogenic acid and cynarin, respectively (Table 3).

Similar permeation results have been found in a study carried out with infusions of *C. cardunculus* (Falé et al., 2013b). A small amount of these compounds, around 10%, were also detected inside the cells (Table 3). Previous studies about chlorogenic permeation though Caco-2 cells indicated that this permeation is highly dependent on the extract composition (Falé et al., 2013a; Zhai et al., 2015).

The influx of the phenolic compounds to the cell interior followed by the efflux to the basolateral compartment allow these compounds to enter the bloodstream and reach different organs, especially the liver where they may inhibit cholesterol biosynthesis through the inhibition of the enzyme HMGR. As shown in Table 3 the decoction of *V. condensata* leaves also inhibits the activity of HMGR with an IC_{50} of 271 μg ml^{-1}. This suggests that the extract can act as a cholesterol reducing agent, probably by a statin-like mechanism. In fact, both main compounds, chlorogenic acid and cynarin, inhibit HMGR activity, even if their IC_{50} values were lower than the commercial hypocholesterolemic drug simvastatin (Table 3). The presence of chlorogenic acid and cynarin in the extract can explain 48% of the IC_{50} value, the remaining activity can be ascribed to the other phenolic acids derivatives, as well as to the flavonoid derivatives containing in the decoction. Calculating the activity of the other phenolic compounds, based on the inhibition value determined for the standards that were structurally similar, 98% of the activity seems to be explained by the identified compounds. Recently, anti-HMGR activity has also been detected in other plant aqueous extracts (Falé et al., 2013b, 2014). Moreover, chlorogenic acid was recognized as an HMGR inhibitor (Falé et al., 2014; Iqbal et al., 2014) and docking studies proved that this molecule can fit within the active site of the enzyme (Navarro-Gonzalez et al., 2014), indicating that this may be one of the procedures by which chlorogenic acid inhibits cholesterol biosynthesis. On the other hand, cynarin, dicaffeoyl quinic acid seems to be a stronger HMGR inhibitor comparatively to the monocaffeoyl quinic acid, chlorogenic acid.

Table 3
Permeation of cholesterol (%) and HMGR activity measured as IC_{50} of the two major phenolic compounds from *Vernonia condensata* decoction. Distilled water was used as a negative control (0% inhibition). Ezetimibe and simvastatin were used as positive controls. All data are presented as the mean ± SD of triplicates.

	Intracellular	Basolateral
Cholesterol permeation		
Control	35.1 ± 7.2^{a}	21.4 ± 3.1^{a}
V. condensata 6 h	10.4 ± 1.4^{b}	23.8 ± 0.5^{a}
V. condensata 24 h	4.4 ± 3.2^{c}	$13.2 \pm 8.6^{a,b}$
Ezetimibe 100 μM	18.9 ± 1.9^{d}	5.2 ± 3.1^{b}
Chlorogenic acid and cynarin permeation		
Chlorogenic acid	13.0 ± 0.2^{a}	26.7 ± 3.0^{b}
Cynarin	12.0 ± 1.1^{a}	15.8 ± 6.1^{a}
HMGR activity		
V. condensata	271.7 ± 24.1 μg ml^{-1}	
Chlorogenic acid	12.9 ± 0.5 μM[e]	
Cynarin	9.1 ± 0.4 μM[e]	
Simvastatin	0.47 ± 0.04 μM[e]	

Superscript letters (a–d) correspond to values in the same column that can be considered statistically different ($p = 0.05$).
[e] Falé et al. (2013b).

In conclusion, beverages prepared by infusion and decoction of *V. condensata* leaves have IC_{50} values 5 times above the toxicity limit and showed antioxidant and anti-HMGR activities, and their bioactive components seem to be stable in conditions similar to the gastrointestinal tract. The main compounds, chlorogenic acid and cynarin, are absorbed through Caco-2 cells monolayers that simulated the intestinal barrier. Our results also suggest that the beverages may reduce dietary cholesterol absorption to a small extent. The present report may explain and provide scientific ground for the molecular understanding of the health benefits of the use of *V. condensata* decoctions to prevent hypercholesterolemia.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contribution

AAA (MSc student) contributed in running the laboratory work, analysis of the data. PLF contributed with supervision in the laboratory work, discussion of the results and contributed also critical reading of the manuscript. LCBC contributed to the collection of plant samples, preparation of the voucher specimen and extracts. RP and LA contributed to the writing and critical reading of the manuscript. MLS designed the study, supervised the laboratory work and contributed to the discussion and critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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