Research Article **IJFNR (2018) 2:14**

International Journal of Food and Nutrition Research
(ISSN:2572-8784)

The potential use of *Mentha x piperita L., Peumus boldus Mol.* and *Baccharis trimera Iless.* extracts as functional food ingredients

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ABSTRACT

We studied the comparative antioxidant and anti-glycation activities of *Mentha x piperita L., Baccharis trimera Iless.* and *Peumus boldus Mol,* in order to evaluate their potential interest as ingredients in functional foods. The total content of polyphenol compounds was determined by Folin-Ciocalteau assay as their antioxidant and anti-glycation capacities, using ABTS and ORAC for the first one and a model with methylglyoxal and bovine serum albumin for the latter. Then, paraoxonase 1 (PON 1) arylesterase activity was measured as well as apolipoprotein A-1 (ApoA-1) structure by SDS-PAGE, in the presence of an oxidative agent and the herbal extracts. Finally, the same procedure was applied to high density lipoprotein (HDL) particles using a Lipoprint kit. Results show that herbal extracts have a considerable amount of total polyphenols, thus a high antioxidant activity and a considerable anti-glycation activity. Furthermore, these extracts restore PON 1 activity as well as the original configuration of apoA-1 and the distribution of HDL subclasses, favouring anti-atherogenic particles. These herbal extracts are interesting targets to use as ingredients in functional foods.

Keywords: Antioxidant, Advanced glycation, Mentha x piperita L., Peumus boldus Mol., Baccharis trimera Iless.,

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How to cite this article:
Agustina Irazusta, Russell Caccavello, Luis Panizzolo, Alejandro Gugliucci, Alejandra Medrano. The potential use of Mentha x piperita L., Peumus boldus Mol. and Baccharis trimera Iless. extracts as functional food ingredients. International Journal of Food and Nutrition Research, 2018; 2:14.

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Introduction

Noncommunicable diseases (NCDs), also known as chronic diseases, are the result of genetic, physiological, environmental and behavioral factors [1]. The main NCDs are cardiovascular diseases (CVDs), cancer, chronic respiratory diseases and diabetes. It is known that heat-processed foods contain a high amount of advanced glycation end products (AGEs) that contribute to the systemic burden of AGEs in the organism. These compounds are known to increase the oxidative stress and inflammation, which are directly related to the development of NCDs, specially CVDs [2,3]. Atherosclerosis represents the main cause for the development of CVDs [4], which are responsible for most NCD deaths which means nearly 17.9 million people annually [1]. Atherosclerosis is a chronic inflammatory reaction of the artery wall that is believed to cause multifocal plaque development [5]. The early stages of the disease involve the recruiting of monocytes to the vessels, which attach to the endothelial cells and then differentiate into macrophages, clean the area and finally turn into foam cells [5,6]. Given the fact that the atherosclerotic plaque is an inflamed area, a high amount of reactive oxygen species are present, which can oxidize many biological molecules and alter their function [5].

High density lipoprotein (HDL) is the name of a highly heterogeneous group of particles composed by lipids and proteins (mainly apoA-I). There is a plethora of different HDL particles, depending on the protein composition which defines their biological functions. It has been studied that the HDL particles that contain a protein called paraoxonase-1 (PON1) are the ones that present more antioxidant, anti-atherogenic and anti-inflammatory capacities as well as better lipid cargo carrying functions. PON1 is secreted by the liver and needs apoA-I associated with apoJ to be fully active [7]. The different HDL particles are classified by increasing size in HDL3c, HDL3b, HDL3a, HDL2b, and HDL2a [8]. Nowadays it is not clearly known which subclasses are the most anti-atherogenic ones, but there is evidence that suggests that the larger ones play an important role, as it was seen that patients with coronary artery disease (CAD) have lower levels of those particles than controls [9–11]. However, former studies indicate that PON1 tends to associate preferably with the smallest HDL_3 particles, which provides them of antioxidant capacity [11]. Furthermore, these small particles are the ones that better uptake cholesterol from peripheral tissues as well as a good anti-atherogenic capacity due to its ability of preventing foam cells formation [12,13]. Many studies [14–16], reveal that patients with CVDs, diabetes and renal failure, either the quantity and antioxidant capacity of PON1 are reduced, this is due to its oxidation [17]. The search for natural alternatives to avoid PON1 oxidation and the effects of such oxidation in the integrity of HDL particles is of great importance when it comes to reduce the development and spreading of CVDs.

In this regard, phytochemicals (flavonoids and other phenolic compounds) are a big group of bioactive compounds present in plants, with a great potential in the prevention of many diseases. This protection is mainly due to their high antioxidant capacity [18]. In Uruguay and its region medicinal herbs are highly used for different purposes;among many species we highlight the frequent consumption of mint (*Mentha x piperita L.*), boldo (*Peumus boldus Mol.*), carqueja (*Baccharis trimera Iless.*) and yerba mate (*Ilex paraguariensis*). Many studies suggest that mint presents a considerable amount of antioxidant capacity which is capable of inhibiting the formation of $NO^{\cdot -}$ and counteract its negative effects [19–21]. Again, it was demonstrated that boldo also presents this type of capacity through different mechanisms of reaction, including protection from iron-induced mitochondrial toxicity [22–24]. Carqueja equally presents antioxidant activity that is capable of reducing the severity of alcoholic hepatotoxicity in rats, as well as presenting anti-inflammatory

properties. Following the same concept, yerba mate also presents a high antioxidant activity [25–28]. Moreover, this herb presents anti-glycation activity capable of inhibit the formation of advanced glycation end products [29] and is also capable of causing the same inhibition within the intestine [30,31]. In addition, it has been proven that aqueous extracts of yerba mate are effective in reducing the oxidation of PON1 [32]. Encouraged by our previous findings that yerba mate presents anti-glycation activity as well as a PON1 protection activity, we turned to the above- mentioned herbs as further interesting study to exploit as a source of bioactive compounds liable to be employed as functional ingredients in foods. The aim of this study was to evaluate the antioxidant activity and especially the inhibition of the oxidation of PON1, as well as the anti-glycation activity of ethanolic and water extracts of mint, boldo and carqueja.

Materials and methods

Chemicals

Folin-Ciocalteu reagent, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2´-azobis (2-amidinopropane) dihydrochloride (AAPH), 3-morpholinosydnonimine (SIN-1) and gallic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). The potassium persulfate ($K_2S_2O_8$) was obtained from J. T. Baker. All other chemicals were of reagent grade. *Mentha x piperita L., Baccharis trimera Iless.* and *Peumus boldus Mol.* powdered dry leaves from commercial sources (Cabral, Montevideo Uruguay).

Extracts preparation

The extracts conditions were selected based on preliminary studies of our group, in which different extracts were compared by their polyphenol quantity and antioxidant capacity. Samples were incubated in a mass:volume ratio of 1:50 in distilled water at 95°C during 3 minutes, or in ethanol 95% at room temperature during 24 hours. The resulting crude extract was first filtered and then lyophilized (BIOBASE™, BK-FD10S) for 96 hours. Finally, they were stored below – 20 °C until further use.

Determination of total polyphenol content

The amount of total polyphenol content in the extracts was assessed by Folin-Ciocalteu assay, according to [33]. Samples (10 µL) were mixed with 200 µL of Na_2CO_3 20% in a 96-well plate, 2 minutes later, 50 µL of Folin-Ciocalteu reagent (1 5^{-1}) were added. After 30 minutes of incubation in darkness, the reading at 750 nm was made using a Thermo Scientific ™ Multiskan ™ FC plate reader. Gallic acid was used as standard and solutions from 0.05-1 mg mL^{-1} were made to obtain a calibration curve. The results were expressed as gallic acid equivalents (GAE) g^{-1} of dried sample.

Antioxidant activity

The antioxidant activity of the extracts was determined by two methods.

Measuring the scavenging activity against ABTS radicals according to [34]. Phosphate saline buffer (PBS): $NaH_2PO_4.H_2O$ and Na_2HPO_4 (5 mM, pH=7.4) was used to dilute the samples, the ABTS reagent and the calibration curve. In order to activate the ABTS reagent (7 mM), 44 µL of potassium persulfate (140 mM) were added to the initial solution, and incubated for 16 hours in darkness. Trolox calibration solutions (0.25-1.5 mM, in assay buffer) were made to obtain a standard curve. 10 µL of sample or standard curve were added to each well of a 96-well plate and 190 µL of ABTS reagent adjusted to 0.7 OD, it was incubated in darkness for 10 minutes and then read at 750 nm using a Thermo Scientific™ Multiskan™ FC plate reader.

Against peroxyl radicals (ORAC) induced by AAPH, using fluorescein as a fluorescence probe as described by [35] and modified by [36]. Herbal extracts were diluted in PBS (75 mM pH=7.4), to a concentration of approximately 0.04 mg mL^{-1}. Trolox calibration solutions (0.1-0.8 nM, in assay buffer) were made to obtain a standard curve. 20 µL of sample or Trolox were

added to a 96-well plate, 120 µL of fluorescein (70 nM) was then incorporated. The first reading was done after 10 minutes of incubation at 37°C in darkness (F0). Then, after the addition of 60 µL of AAPH, a reading per minute was carried out for a total of 104 minutes (excitation ʎ= 485 nm, emission ʎ= 520 nm), using a Thermo Scientific™ Varioskan™ Flash spectral scanning multimode reader.

Results are expressed in µmoles of Trolox equivalents (TE) g^{-1} of dried sample in both assays.

Anti-glycation activity

The anti-glycation activity was determined measuring the fluorescence intensity from the *in vitro* glycation of the protein following the methodology proposed by [37], with some modifications. The model used was the glycation reaction between bovine serum albumin (BSA) and methylglyoxal (MGO), testing the herbal extracts as inhibitors. Aminoguanidine was used as a reference inhibitor of the reaction; a standard curve was made with 5 concentrations between 0.0005 and 0.1 mol L^{-1}. Concentrations between 0.01-1 mg mL^{-1} of herbal extracts were incubated in the presence or absence of 5 mM of MGO and 1 mg mL^{-1} of BSA. Half of them were stored at -20°C right after they were prepared (Day 0) and the other half were incubated at 37°C for 7 days and then stored at -20°C until analysis (Day 7).

The fluorescent AGEs were determined spectrofluorometrically (Thermo Scientific™ Varioskan™) using fluorescence intensity at an excitation wavelength of 340 nm and emission wavelength of 420 nm. The inhibition percentage of the glycation reaction was calculated as follows:

$$\% \text{ inhibition of glycation} = 1 - (F(\text{sample}) - F(\text{IFC}))/(F(C+)) \times 100$$

In which F (sample) is the fluorescence of the sample, F (IFC) is the intrinsic fluorescence control (the fluorescence of the herbal extract alone) and F (C+) is the fluorescence of the positive control (BSA + MGO).

Results are expressed as IC50 values (concentration of sample needed to inhibit 50 percent of the glycation reaction).

HDL preparation

The HDL (d= 1.063- 1.210 g L^{-1}), was purified by sequenced ultracentrifugation from a pool of human serum from healthy volunteers (n=30) [38]. Participants signed an informed consent. The study was approved by Touro University California IRB # 2013-069.

HDL oxidation

Arylesterase activity of paraoxonase 1 (PON1) was measured following the product phenol (at 270 nm) from the phenyl acetate catalysis [32].

HDL was diluted in 20 mM of Tris buffer pH= 8 containing 1 mM of $CaCl_2$, to a final protein concentration of 1 mg mL^{-1}. It was incubated in presence or absence of a hydrophilic free radical initiator AAPH (final concentration of 2 mM), or nitric oxide (NO*) donor SIN-1 (final concentration of 0.1 mM), at 37ºC for a period of 3 hours. At the same time, samples were incubated in the presence or absence of the herbal extracts (final concentrations between 0.01 and 0.5 mg mL^{-1}). After incubation, samples were snapped frozen at -80°C. Immediately before the assay, 200 µL of phenyl acetate solution (1 mM) were added to each well to start the reaction. To follow the kinetics of the reaction, six readings in 1 minute total were made at 270 nm in a BioTek®, Synergy H1 plate reader.

SDS-PAGE

In order to determine possible structural changes in the apoA-1 (anti-atherogenic protein of HDL complex) such as in the molecular weight in oxidative conditions and in the presence of aqueous herbal extracts, 20 µg of sample were loaded in each lane of a 10% BioRad Mini Gel III, in reducing conditions. Finally, the gels were stained with Coomassie Colloidal Blue. The densitometric analysis was made using GS-900 BioRad equipment with the proprietary software (Hercules, CA, USA).

Lipoprint® analysis

25 µL of serum and 35 µL of samples (prepared to assess the inhibition of oxidation of PON-1), were used for the structural analysis of the HDL complex under native conditions using the Lipoprint® HDL Subfractions* Test kit, from Quantimetrix (Redondo Beach, CA, USA).

Statistical analysis

All the extracts were made in triplicate and total polyphenol content and antioxidant activities were determined in three replicates. Results were presented as mean values ± standard deviation (SD). Statistical analysis was performed using Infostat [39]. One-way analysis of variance (ANOVA) followed by Tukey's test were used to identify significant differences between extraction conditions. Differences were considered as statistically significant when $p < 0.05$.

Results and Discussion

Total polyphenol content of herbal extracts

The quantity of phenolic compounds was measured following the protocol of the Folin-Ciocalteu assay. The water herbal extracts showed a higher amount of total polyphenols compared to the ethanol 95% (figure 1). Furthermore, the two extracts with the highest quantity of these compounds were the aqueous extracts of mint and boldo with 86 ± 8 and 88 ± 8 mg GAE g^{-1} of dried sample, respectively. There are no significant differences between the both ($\alpha \leq 0.05$). This relationship in the quantity of polyphenolic compounds among aqueous extracts is also seen in the work done by [40]. Considering that Folin- Ciocalteu reagent reacts with hydroxyl groups present on the aromatic rings of polyphenols [41], it is reasonable to think that a more effective extraction of these type of compounds would be in water, due to their hydrophilic nature [42]. Moreover, these high values of concentration of polyphenols also reflex the maintenance of their structure in spite of the high temperature of the medium (95°C), and this factor might even improve the total polyphenol extraction [43]. No significant difference ($\alpha \leq 0.05$) was found in the ethanolic extracts; boldo and carqueja were the ones that presented more polyphenolic compounds, with 61 ± 6 and 53 ± 5 mg GAE g^{-1} of dried sample respectively.

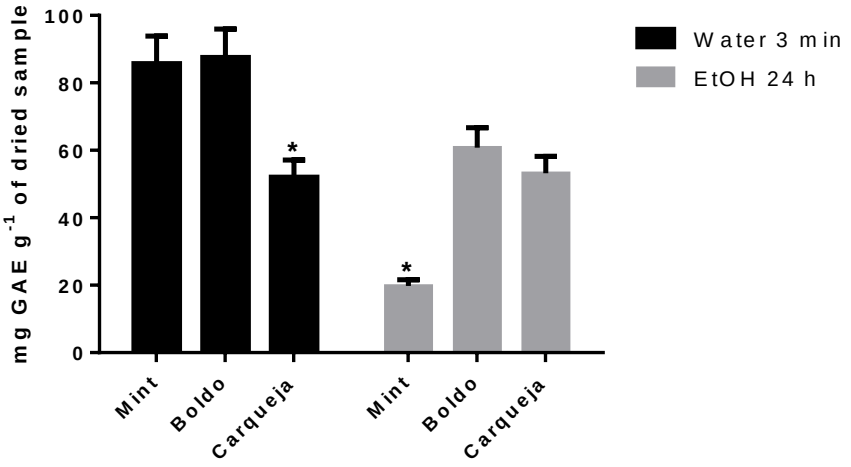


Figure 1: Content of total polyphenols present in the aqueous and ethanolic extracts determined by Folin-Ciocalteau assay. Results are expressed in mg GAE g^{-1} of dried sample. Asterisks indicate significantly different values.

Antioxidant activity against ABTS$^{\bullet-}$ and peroxyl radicals

The antioxidant capacities of the different herbal extracts varied between 30 and 75 µmol TE g^{-1} of dried sample of scavenging activity against ABTS radicals, and varied between 11 and 116 µmol TE g^{-1} of dried sample against peroxyl radicals (ORAC) (Figure 2 A and B, respectively). The ethanolic extract of mint presented less antioxidant activity in both assays. Boldo was the herb that showed more antioxidant activity against ABTS radicals either in water or ethanol. Regarding the scavenging activity of the samples against peroxyl radicals, in the aqueous extracts the ones that presented more antioxidant activity were mint and boldo, showing no significant differences (α≤ 0.05). These results agree with the ones obtained for the total polyphenol content (figure 1), showing a directly proportional relationship, being the samples with more antioxidant capacities the ones with more of this type of compounds and vice versa. This suggests that polyphenols are the main compounds responsible for the antioxidant activity of the extracts. However, carqueja was the extract with higher antioxidant activity of the ethanolic extracts against peroxyl radicals, not corresponding with the relation observed either in quantity of total phenolic compounds nor in the activity against ABTS radicals. This suggests that this extract may contain other types of compounds (not polyphenols) responsible for this high antioxidant activity against peroxyl radicals.

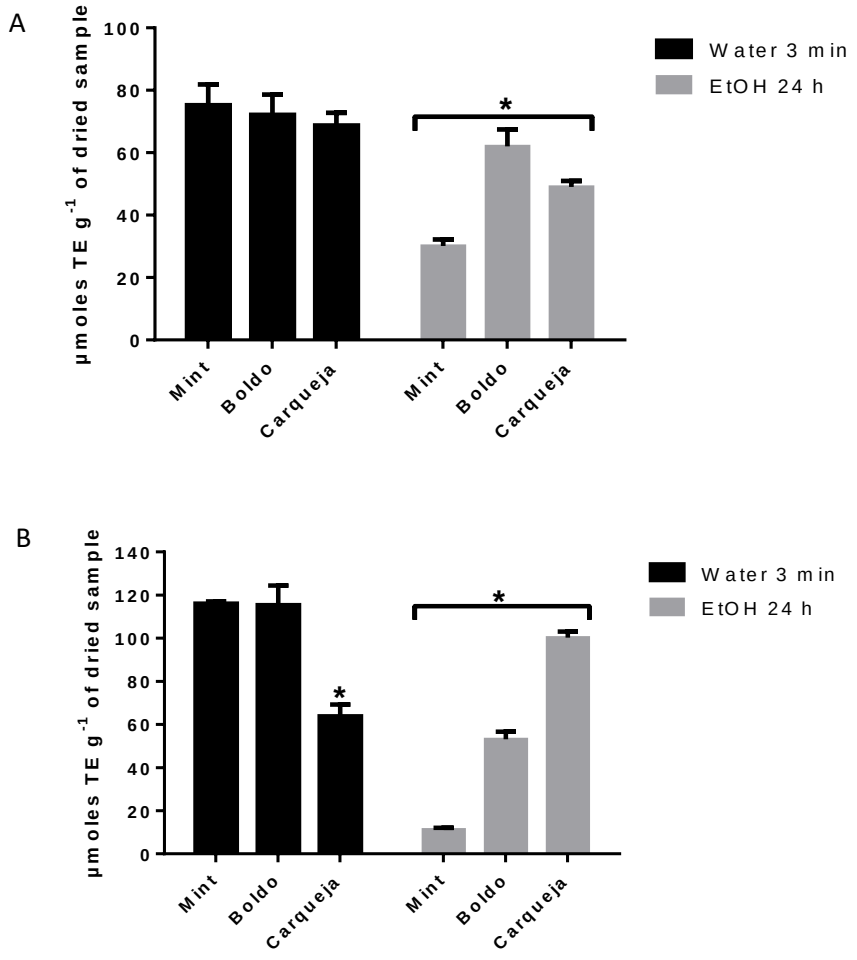


Figure 2. Antioxidant capacity of the aqueous and ethanolic extracts measured against ABTS radicals (A) and peroxyl radicals (B), expressed in µmoles TE g^{-1} of dried sample. Asterisks indicate significantly different values.

Anti-glycation activity of the herbal extracts

Our results also showed that the herbal extracts efficiently inhibited AGE formation. If we compare the IC50 values of the extracts with the one obtained for the inhibition standard aminoguanidine, the first ones have a lower anti-glycation activity than the latter. However, considering that in a cup of tea (4 grams of dried herb in 200 mL of hot water) approximately 1 gram of extract is consumed, the concentration of it in the intestine will be higher than that needed to inhibit 50% of the glycation reaction. It can be concluded that the concentration of natural extract needed to cause an inhibition of the glycation reaction is very low and can easily be consumed in a cup of tea. Ethanolic mint presented a lower IC50 value than the aqueous extract (43 ± 3 µg mL^{-1}) which means that the first has a higher anti-glycation activity than the latter. Concerning boldo, there are no significant differences between both extracts. Ethanolic carqueja showed no significant differences with ethanolic boldo. The extract with less anti-glycation activity was aqueous mint due to its high value of IC50 (58 ± 5 µg mL^{-1}). On the contrary, the most anti-glycation extract is aqueous carqueja with an IC50 of 14 ± 1 µg mL^{-1}. These results suggest that the anti-glycation activity depend on polyphenols like the antioxidant capacity, but in the case of ethanolic carqueja, the type of polyphenols that it contains may be more effective in the inhibition of the glycation reaction.

In summary, we showed that hot water extracts seem to be the ones with better antioxidant and anti-glycation capacities; this is an advantage because it is environmentally friendly and also has a very low cost.

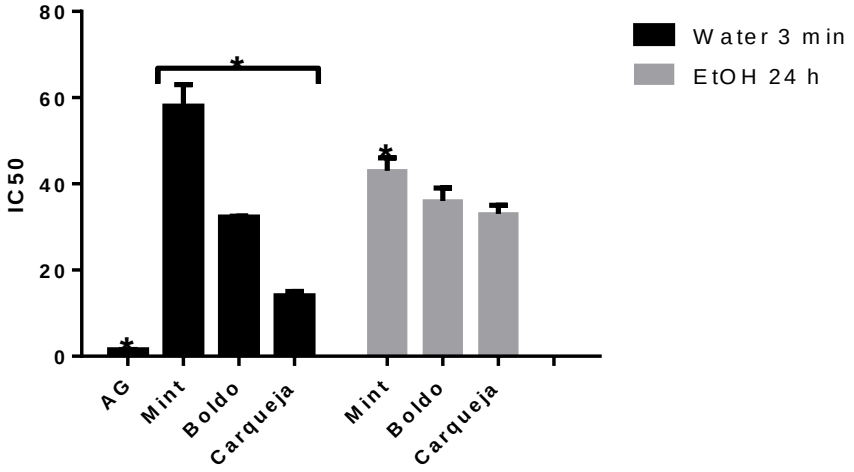


Figure 3. IC50 values of antiglycation capacity of the herbal extracts obtained incubating each of them in the presence of methylglyoxal and BSA for 7 days at 37°C. Aminoguanidine (AG) was used as the reference inhibitor. Results are expressed in µg mL^{-1}. Asterisks indicate significantly different values.

Protection of PON1 against oxidation by the herbal extracts

In order to assess the protection of PON1 activity by prevention of its oxydation, the next assays involved the incubation of purified HDL with different concentrations of herbal extracts, in the presence of two different oxidative agents as shown in figure 4 (A and B).

The results for the protection of PON-1 activity by the extracts (final concentration of 0.05 mg mL^{-1}) were highly encouraging (figure 4). In the presence of AAPH (figure 4, A), aqueous

extracts had a maximum of 90% and ethanolic extracts of 96%, which are considerably high values of recovery of PON1 activity. Something similar occurred for SIN-1 (figure 4, B), in which 122% was the maximum for aqueous extracts and 117% for ethanolic extracts, even improving PON1 activity considerably. In all cases boldo was the less effective herb in recovering PON1 activity, the opposite occurred with mint, reaching values of 122% of inhibition in the presence of SIN-1, not only protecting completely the enzyme, but also improving its activity by a 22% in that case. As commonly known, SIN-1 is an oxidative agent which releases NO, superoxide and peroxynitrite under physiological conditions. These results suggest that the antioxidant capacities of mint extracts are more effective preventing the nitration of the tyrosines present in the protein. Furthermore, this anti-nitration activity from mint ethanolic extract seems to be independent from its antioxidant capacity, due to the fact that that extract was the one with less antioxidant capacity, and also presented a poor anti-glycation capacity.

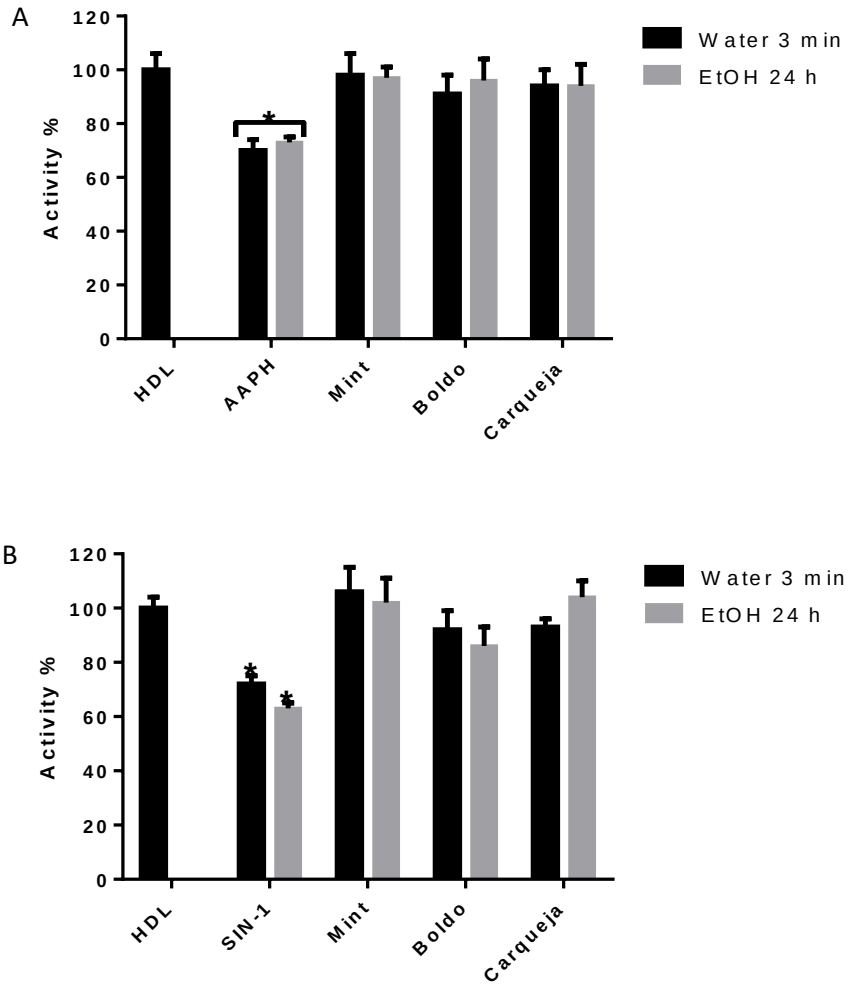


Figure 4. Percentages of PON-1 activity when HDL was co-incubated for 3h at 37°C with the herbal extracts (concentration of 0.05 mg/mL) and AAPH (A) or SIN-1 (B). Control PON1 activity (100%) was measured incubating HDL alone (HDL), the loss of PON1 activity due to oxidation was obtained by incubating HDL in the presence of AAPH or SIN-1 (showed as either AAPH or SIN-1). The recuperation of PON1 activity was measured incubating HDL in the presence of oxidative agent and herbal extract (showed as Mint, Boldo or Carqueja). Asterisks indicate significantly different values.

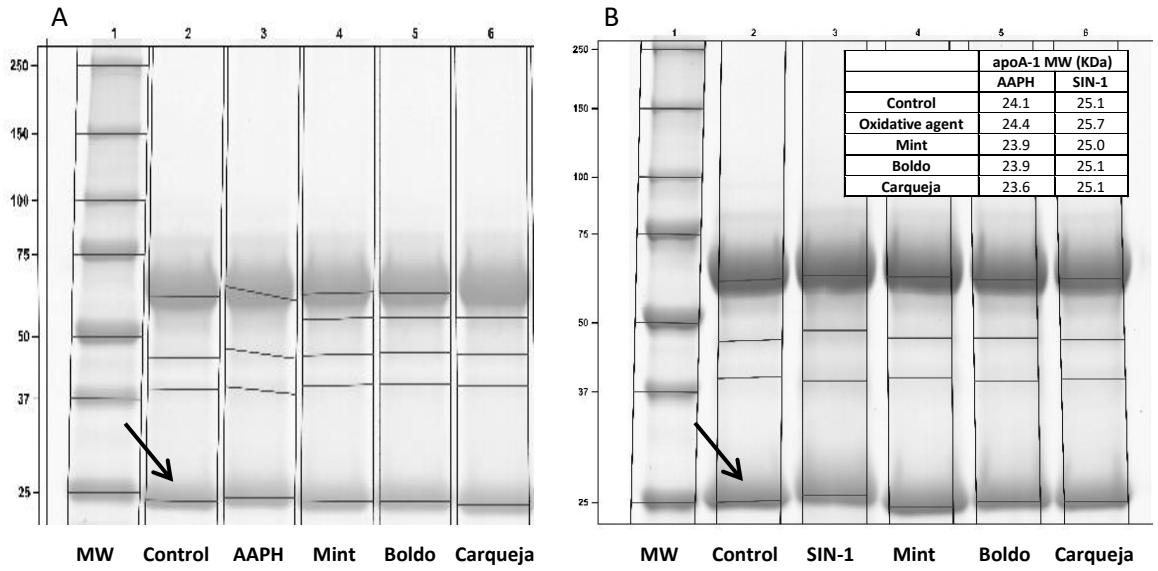
In conclusion, we demonstrate that these extracts inhibit the formation of AGEs and also considerably reduce the oxidation of PON1 in the presence of AAPH and SIN-1, even improving its activity in some cases. These characteristics raise their value and interest as ingredients in future functional foods.

To further dissect the mechanisms involved in the protection we explored the effects of oxidants and herb extracts on apoA-1.

Protection of ApoA-1 structure against oxidation

ApoA-1 is the major protein present in the structure of HDL complex, it is responsible (together with ABCA1) of initiating the cholesterol efflux from macrophages to originate de HDL particles [44]. Also, PON1 needs apoA-I associated with apoJ to fully activate [7]. It has been shown that this molecule also presents anti-atherogenic functions of relevant interest [11]. The conservation of its structure is important for the maintenance of its activity, so the next step was to evaluate the influence of the oxidative agents and herbal extracts in the conformation of this protein.

For the assay with AAPH as oxidative agent, a concentration of aqueous herbal extract of 0.01 mg/mL was used but in the case of SIN-1 the concentration used was of 0.05 mg/mL. The results are shown in figure 5 (table 5).



	apoA-1 MW (KDa)	
	AAPH	SIN-1
Control	24.1	25.1
Oxidative agent	24.4	25.7
Mint	23.9	25.0
Boldo	23.9	25.1
Carqueja	23.6	25.1

Figure 5: SDS-PAGE gels. In each lane were loaded 20 µg of sample of HDL incubated in the presence of AAPH (A) and SIN-1 (B) and aqueous herbal extracts for 3h at 37°C. Line 1: molecular weight, line 2: HDL control (incubated alone), line 3: HDL + AAPH or SIN-1, line 4, 5 and 6: HDL + AAPH or SIN-1 + herbal extract (Mint, Boldo, Carqueja). In the attached table are shown the values of apoA-1 molecular weight (KDa) obtained by densitometric analysis of the SDS-PAGE gels. Arrows indicate the apoA-1 protein.

In figure 5, a slight change in the structure of apoA-1 (MW) is seen either against AAPH or SIN-1. Interestingly, when the aqueous herbal extracts were incorporated, this change seems to be reverted, returning to its original size.

As shown in the table in figure 5 and confirming what is seen in the gels, the molecular weight of apoA-1 is clearly affected by the presence of the oxidative agent, increasing its weight from 24.1 KDa to 24.4 KDa in the case of AAPH and from 25.1 KDa to 25.7K Da in the case of SIN-1. As we see in figure 5, when aqueous herbal extracts were incorporated the molecular weight values returned to their originals. These results show

that the extracts have the activity to recover the original structure of apoA-I. Following these encouraging results and in order to dissect the mechanisms involved in the protection, we explored the effects of oxidants and herb extracts on the HDL subclass distribution.

HDL subclass profile restructuration in the presence of the herbal extracts

Nowadays it is not clearly known which subclasses of HDL are the most anti-atherogenic ones, but there is evidence that suggests that the larger HDL_2 particles play an important role for instance, patients with coronary artery disease have lower levels of those particles than controls [9–11]. Precisely, herbal extracts display a very interesting beneficial effect on HDL particles subclass distribution when exposed to AAPH. As shown in figure 6, purified HDL do not contain small HDL particles but when exposed to oxidation, however, those particles suffer a restructuration in which smaller ones are created from the larger ones. Once the extracts are added, the HDL subclass profile returns to the original one. These results indicate that the herbal extracts have the ability to restore the HDL subclasses to its original distribution. If we put together these results with the beneficial effects on PON-1 activity (figure 4), as well as on apoA-1 structural changes our data show that our herbal extracts have significant effects in protecting HDL particles from structural and functional damage by oxidation.

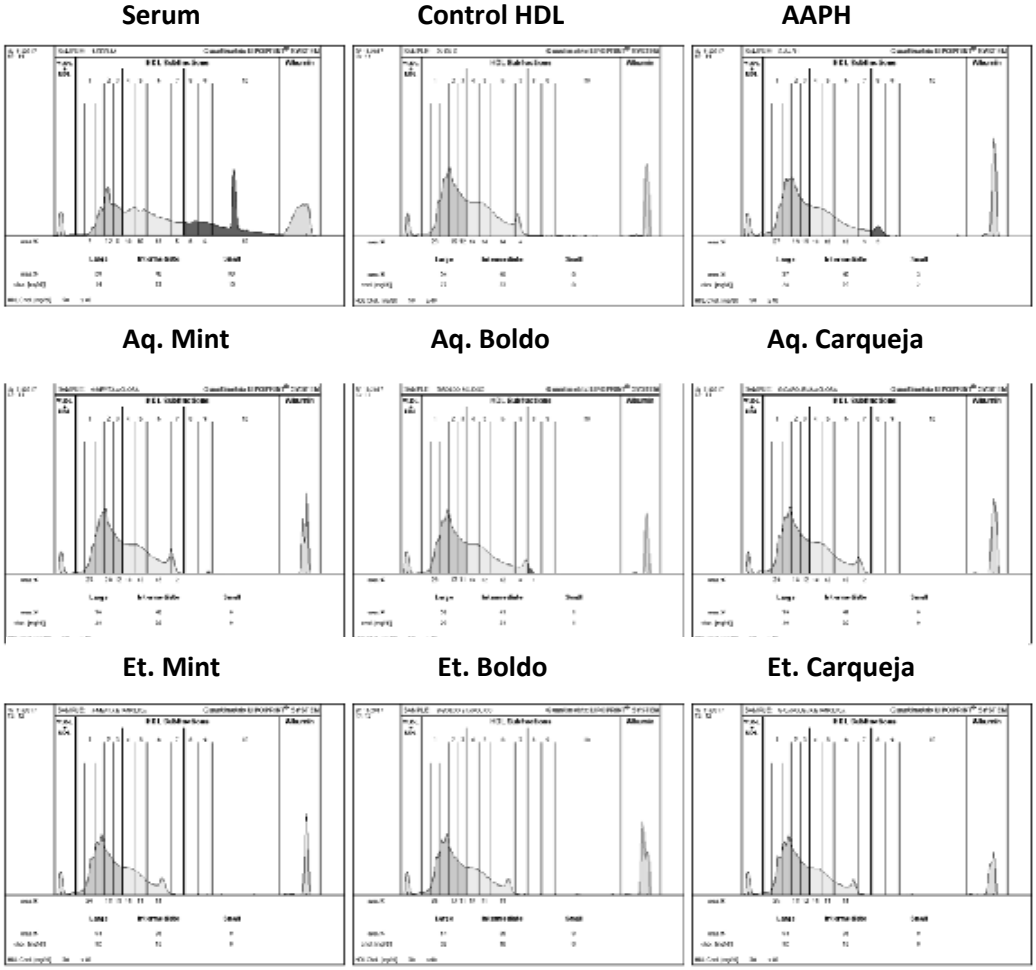


Figure 6. Representative subclasses profiles of HDL for all cases. First profile corresponds to non-treated serum, the second to purified HDL. Results obtained incubating HDL with AAPH or SIN-1 for 3h at 37°C in the presence of herbal extract, then running on Lipoprint native gel. Power source

was set to 3mA per tube, to 500V for 50 minutes. Medium grey represents larger particles, light grey medium size particles and dark grey the smaller ones.

The changes in HDL particle subclass distribution were not the same in the presence of SIN-1, given that the same profile was seen in all cases. This data suggests that oxidation (mostly lipids) produces changes in the population of HDL while nitration (mostly of Tyr in proteins) does not have the same effects.

Conclusion

According to all the data presented in this research, we first conclude that the water and ethanol herbal extracts of the hebs studied prevent PON-1 inactivation by nitration and oxidation. Hot water extracts displayed the highest potency as antioxidant and anti-glycation agents.

Herbal extracts restore the original configuration of apoA-1 as well as the distribution of HDL subclasses, favouring anti-atherogenic particles. Using the HDL model we determined that two different mechanisms that are key in the pathology of chronic diseases, oxidation of lipids and peroxynitrite nitration may be attenuated by these herbal extracts. Secondy all extracts also show anti-glycation activity which prevents the formation of AGEs which are also key players in CVD. Therefore, herbal extracts made with *Mentha x piperita L., Baccharis trimera Iless.* and *Peumus boldus Mol.* which are commonly consumed in Uruguay and other regions in South America, are a protective source against oxidation and glycation, and are interesting targets for bioactive compounds to use as functional ingredients in foods.

Acknowledgements

The authors acknowledge the financial support from the Plan de Desarrollo de las Ciencias Básicas (PEDECIBA), Comisión Central de Investigación Científica (CSIC). Universidad de la República, Uruguay and Touro University California

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