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# In Vitro Bioaccessibility and Antioxidant Capacity of Extracts Obtained from Boldo Leaves (*Peumus boldus*) For Its Application as a Functional Ingredient †

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**Abstract:** The aim of this study was to obtain an extract from boldo leaves (*Peumus boldus*) with antioxidant properties, studying its bioaccessibility and potential application in the development of orange biscuits. The extraction conditions were defined by performing a factorial design, working with two variable factors: extraction time and solvent concentration (96° ethanol and water). Total phenolic compounds (TPC) content was determined by Folin-Ciocalteau method, and antioxidant capacity (AC) against ABTS•+ radicals. On the selected extract, phenolic compounds were identified by UHPLC. To evaluate its application as a functional ingredient, TPC and AC were determined to orange biscuits with different extract content. Furthermore, bioaccessibility was determined by applying the standardized static in vitro digestion method Infogest. Results showed that 1 h extraction time, using water as solvent at 70 °C had the highest content of total phenolic compounds (218.83 ± 25.91 mg eq GAE/g of extract) and the best antioxidant potential being the AC of 720.56 ± 15.00 μMol eq TE/g of extract. Gallic acid, chlorogenic acid, rutin and catechin were identified as the main phenolic compounds on this sample. Regarding the use of boldo extract in the development of a functional orange biscuits, a significant increase in the content of TPC and AC was observed compared to a control biscuit (without extract addition). The study of bioaccessibility evidenced a reduction of 87% in the content of phenolic compounds. Thereby, further studies in strategies of encapsulation of the extract are needed. In conclusion, boldo extract was found to be an ingredient with potential functionality for its use in food processing.

**Keywords:** *Peumus boldus*; functional ingredient; functional food, bioaccessibility

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## 1. Introduction

Due to the current pace of life, the last few decades have seen a transformation in eating habits caused by the lack of time to cook and high consumption of food with sugar, fat and sodium that are now available and accessible everywhere. There is increasing scientific evidence that poor nutrition together with certain behavioural factors contributes to the development of NCDs [1]. New generations of consumers are becoming conscious about their diet, searching the market and demanding that the industry provide new foods that contribute to their health and wellbeing [2].

In recent times there has been a notable increase in the use of medicinal herbs, occupying a very important consumption in the world of alternative medicine [3]. For this reason, scientists are currently interested in studying herbs that have been used throughout history [4], to incorporate them into new formulations and develop new functional

foods. One herb widely used in Uruguay and Latin America is Boldo (*Peumus boldus Mol)*. It is known for its properties for liver treatment, due to boldine [5]. Boldo leaves have been shown to possess more than 30 compounds such as quercetin glycosides, kaempferol derivatives, phenolic acids, proanthocyanidins, among others [6,7]. In this study [8], they compared the content of phenolic compounds, antioxidant capacity and anti-inflammatory activity of commercial herbal infusions, and determined that boldo infusions had a higher content of phenolic compounds and antioxidant capacity than other samples.

The aim of this work was to provide novel information regarding to in vitro bioaccessibility of the bioactive compounds present in a extract from boldo leaves (*Peumus boldus*) and studying the potential application in the development of orange biscuits.

## 2. Materials and Methods

### *2.1. Samples of Boldo Leaves*

Were used dehydrated leaves of *Peumus boldus Mol*. for commercial use Cabral® (Montevideo, Uruguay). The leaves was ground with a domestic mill at a power of 130 W and a frequency of 50 Hz for 3 min, reaching a fine particle size. The crushed samples were stored in glass jars at 4 °C.

### *2.2. Experimental Design*

An experimental design [9] (central compound design) was carried out according to the conditions of Table 1, based on a response surface model, factorial design. The experimental design consisted of four trials (systems 1, 2, 3, and 4), 2 factors with 2 levels, and three central points to estimate the experimental error (systems 5, 6, and 7). The variables studied were extraction time (t, expressed in hours) and ethanol: water ratio (r, expressed in %). The two levels studied were 30/70/1 and 30/70/2% water /% ethanol/time respectively. Three central points were obtained using the following conditions: 1.5 h, 65/35% water/ethanol. Each system was contained 8.0g of ground sample, 0.5 g of citric acid, and 400 mL of solvent. The samples were put in a water bath at 70 °C, with agitation following the time specified for each system. Then the extracts were filtered by vacuum filtration, using filter paper. The Samples that contain hydroalcoholic solvent, after having been filtered, Ethanol was evaporated in rotary evaporation at 60 °C. All samples were frozen at -20 °C for 96 h and freeze-dried for 120 h.

### *2.3. Total Fenolic Compound and Antioxidant Capacity*

Phenolic compounds were determined in samples by Folin-Ciocalteau method as described by Fernández-Fernández. et al. [10]. Gallic acid was used as reference with 5 concentrations ranged between 0.05 and 1 mg/mL. 10 μL of standard solution or sample and 200 μL of 20% sodium carbonate ($Na_2CO_3$) were mixed. After 2 min, 50 μL of Folin-Ciocalteu (1/5 dilution) reagent was added. The mixture as ept in darness for 30 min and absorbance at 750 nm was determined using the MULTISKAN FC-Thermo Scientific plate reader.

The Overall antioxidant capacity of the samples was performed employing the ABTS methodology described by Fernández-Fernández. et al. [10], with some modifications. The radical ABTS• + (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was prepared by mixing 10mL of ABTS (7 mM) reagent and 176 μL of potassium persulfate (140 mM), keeping the mixture in the darkness for 16 h. TROLOX standars solution were used for calibration curve withconcentrations between 0.25 and 1.5 nM. A 96-well microplates was used, 10 μL of sample were added with 190 μL of ABTS • + solution previously adjusted to an absorbance of 0.7 AU in darkness, afterwards, absorbance was measured using the MULTISKAN FC-Thermo Scientific plate reader at 750 nm.

*2.4. Identification Compounds by UHPLC*

The samples were analyzed by liquid chromatography procedure described by Fernández-Fernández. et al. [10], with some modifications. An UltiMate 3000 UHPLC equipment (Thermo Scientific), with a diode array detector, and a Thermo Scientific BDS Hypersil C18 reversed-phase column was used, with a flow of 1 mL/min. The mobile phase was methanol (A), acetonitrile (B), phosphoric acid pH 2.81 (C). The system was run with the following gradient elution program: 0 min, 5% A/95% C; 10 min, 10% A/10%B/80% C; 20 min, 20% A/20% B/60% C; 40 min, 20% A/20% B/60%; 45 min, 100%B; 50 min 100%B; 55 min 5%B/95%C. The duration of each run was 55 min. The sample used is the same at Section 2.3. The injection volume was 20 µL. Six standards were used as reference compounds: gallic acid, caffeic acid, chlorogenic acid, quercetin, catechin and boldine. The identification of each compound in analyzed samples was performed by comparison with retention times and their absorbance at 290 nm, while catechin was detected at 370 nm. The data were processed using Dionex Chromeleon 7.1 SR2 software.

*2.5. Bioaccesibility and Bioactivity Assays*

The selected extract was subjected to simulated digestion to obtain the bioaccessibility and bioactivity as, described Fernández-Fernández. et al. (2021) [11]. The digestion process was divided into three stages: first is the salivary stage, which includes the addition of $\alpha$-amylase enzyme (3.9 unit/mL), the system was incubate for 5 min; the gastric stage is the second, it consists of a closed system, where the pH was adjusted to 2 with 1M HCl. 1.5 mg of the enzyme pepsin was added and it was left to incubate for 90 min. The third and last stage is the duodenal one, which is also a closed system in which the pH was adjusted again to 7 using 1 M $NaHCO_3$. Then 9.2mg of the enzyme pancreatin and 55.2 mg of bile were added, and finnally it was incubated for 150 min. The incubations were carried out at 37 ± 1 °C with heating plates and with constant shaking at 250 RPM. It was centrifuged for 20 min at 9500 rpm, and the supernatant was freeze-dried for 96 h. After freeze-dried, these samples were used to determine total phenolic compounds and ABTS antioxidant capacity, as described in Section 2.3.

*2.6. Biscuits Preparation*

It was used the procedure described by Fernández-Fernández. et al. (2021) [11] to formulate the biscuitswith some modifications. Four samples of orange biscuits enriched with boldo leaf were prepared with different percentages of flour/boldo extract: sample 1: 46.54/0%; sample 2: 46.14/0.4%; sample 3: 45.64/ 0.9 %; sample 4: 45.14/1.4%. For elaboration the flour and the boldo extract, were mixed in a mixer at a power of 130 W and a frequency of 50 Hz for 5 min to homogenize. After that, the rest of the ingredients were added, forming a homogeneous dough. The cookies were cut with circular molds of 4.0 cm in diameter and with a height of 0.5 cm, to then be baked, in a Xion brand electric oven, for 10 min at 160 °C.

*2.7. Fenolic Compound and Antioxidant Capacity of Biscuits*

Samples of each formulation were triturated, weighed by 100mg and 100 µL of DMSO were added. After that, 1200 µL phosphate buffer saline (PBS) 5 mM pH = 7.4 were added for better polyphenols extraction.The content of phenolic compounds and ABTS antioxidant capacity of the biscuits was determinedfollowing Section 2.3 above.

*2.8. Statistical Analysis*

All analyses was performed by triplicate. The results were treated by analysis of variance (ANAVA), using the Infostat, Minitab and Graphpad Prism software. Differences between groups were consider significant when $\alpha < 0.05$. Where significant differences existed, Tukey comparasion of means was applied at the 0.05 level of significance.

## 3. Results and Discussions

### *3.1. Antioxidant Compound of Extract*

Table 1 shows the results for phenolic compound content and antioxidant capacity. In a study published by Soto et al. (2013) [12], they extracted boldo samples from tea bags, using 1 g/50mL water at 90 °C for 5 min, and obtained values of 34.3 ± 2.1 mg GAE/g dry sample. Similar results can be seen in the work by Iazusta et al. (2018) [13] where carried out an extraction (1/50) on a commercial sample of boldo leaves using water at 95 °C for 3 min, and obtained a phenolic compound content of 88 ± 8mg GAE/g dry sample. Our extracts presented between 3 and 6 times more phenolic compounds than the aforementioned studies. The ABTS antioxidant capacity results show similar values to those obtained by Speisky et al., (2006) [14] in boldo tea samples extracted with water at 95–100 °C for 5 min, 898 µMol eq TE/mL herbal tea. The extract system selected was system 3, as it was a simple and environmentally friendly extraction using water as a solvent at a temperature of 70 °C and an extraction time of 1 h. In terms of total phenol content, system 3 did not show significant differences with the rest of the systems, obtaining 218.83 ± 25.91 mg eq GAE/g extract dry, but in terms of antioxidant capacity, there were significant differences between the systems, being system 3 the one that showed differences only with system 7 but not with the rest of the systems. A value of 720.56 ± 15.00 µMol eq TE/g extrac dry was obtained.

### *3.2. Characterization of the Selected Extract*

By UHPLC, four phenolic compounds have been identified in boldo leaf extracts: gallic acid, chlorogenic acid, rutin, and catechin (Figure 1). These results are similar with other studies where these compounds were also found in extractions using water as a solvent [15,16]. It's reported that boldo extractions contain caffeic acid and boldine [15,16]. In this work, we couldn't identify caffeic acid and boldine compound probably due to extraction temperture, whereas other studies used higher temperatures (over 80 °C) than the used in the present work (70 °C) [15,16]. Several authors mention that boldine has a low contribution to the antioxidant activity in boldo extracts, being the rest of the phenolic compounds the ones with the highest activity [17,18].

**Compounds identified in this study have beneficial properties for human health.** Gallic acid has been reported with benefits to suppress lipogenesis, reduce plasma glucose, insulin and triglyceride levels [19,20]. Coffee beans have shown health benefits, which are attributed to chlorogenic acid, this being one of the main compounds present [21]. Recent studies have shown that the ingestion of 300–600mg of chlorogenic acid present in 1~2 cups of coffee, improves insulin resistance, lipid oxidation and brain function [22]. Rutin has shown beneficial properties against cardiovascular diseases, neurodegenerative disorders, and improves lipid profile [23]. Catechins have many beneficial properties for human health, anti-cancer effects, anti-obesity, anti-diabetic, this is due to their antioxidant and prooxidant power intervening against reactive oxygen species [24].

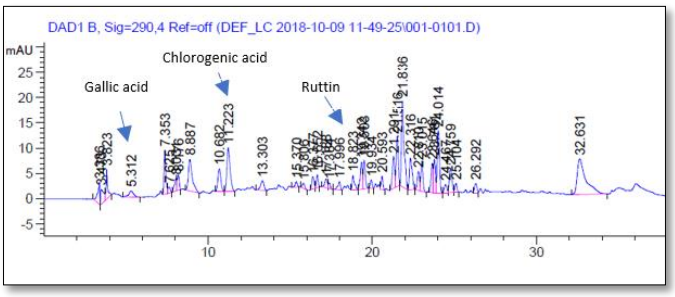



**Figure 1.** Chromatograms obtained at a wavelength of 290 nm. Gallic acid ($t_R$: 5312), chlorogenic acid ($t_R$: 11,223) and rutin ($t_R$: 18,823).

**Table 1.** Total phenol compound, antioxidant capacity and composition of the systems with their respective times.

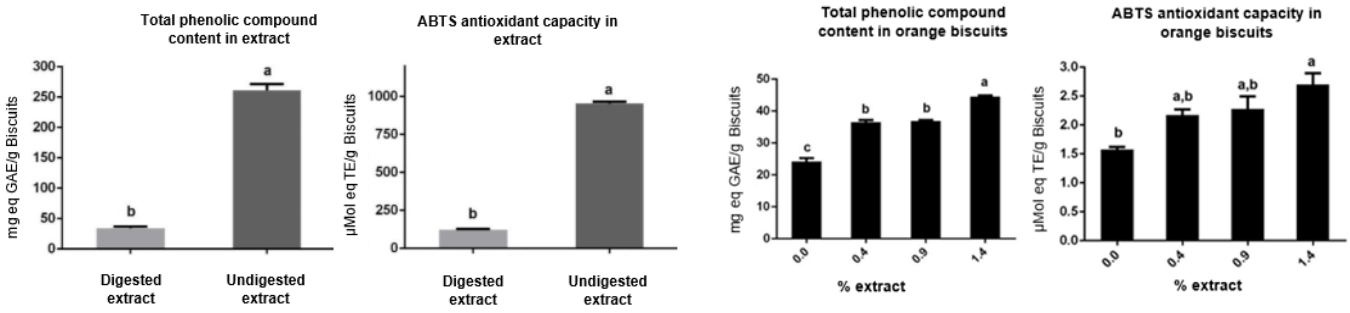
| Systems | Total Phenol Compound mg eq GAE/g Extract Dry | Antioxidant Capacity µMol eq TE/g Extrac Dry | % Water | % Ethanol | Time (h) |
|---|---|---|---|---|---|
| System 1 | 215,79 ± 12,00 a | 864,99 ± 24,49 a | 30 | 70 | 1 |
| System 2 | 216,91 ± 30,99 a | 792,37 ± 57,71 a | 30 | 70 | 2 |
| System 3 | 218,83 ± 25,91 a | 720,56 ± 15,00 a,b | 100 | 0 | 1 |
| System 4 | 213,30 ± 39,14 a | 732,97 ± 33,17 a,b | 100 | 0 | 2 |
| System 5 | 173,76 ± 15,21 a | 883,12 ± 54,95 a | 65 | 35 | 1.5 |
| System 6 | 193,09 ± 21,31 a | 603,80 ± 31,54 b,c | 65 | 35 | 1.5 |
| System 7 | 196,89 ± 8,29 a | 514,70 ± 26,89 c | 65 | 35 | 1.5 |

Note: All results are expressed as the average ± standard error, different letters in the same column indicate significant differences according to Tukey, $p < 0.05$.

### *3.3. Bioaccessibility Study of the Extract before and after In Vitro Digestion*

Figure 2 show total phenolic content and ABTS antioxidant capacity in the undigested and digested extract. It can observe a lower presence of phenolic compounds and antioxidant capacity in the extract after digestion. Nevertheless, results show some antioxidant and phenolic compounds that supported the digestion process. The results obtained are consistent with several studies, wherelosses of phenolic compounds and antioxidant capacity in samples of Yerba mate (ilex paraguatiensis) [25] and Citrus pomaces [11] are reported.

In this stady a reduction of 87% was obtained with respect to the content of phenolic compounds. It can be observed that the compounds involved in the extract are very sensitive to the digestion process and only 13% was bioaccessible. However, a low antioxidant capacity in vitro does not always represent a low capacity in vivo, according to [26] some compounds with low antioxidant capacity in vitro, after their metabolization into simpler compounds, may contribute to the antioxidant capacity of plasma.




(**A**) (**B**)

**Figure 2.** (**A**) Total phenol content and ABTS antioxidant capacity in the undigested and digested extract. Difference letters (a and b) indicate significant differencecs (Tukey, $p < 0.05$). (**B**). Total phenol content and ABTS antioxidant capacity in oranges biscuits. Difference letters (a and b) indicate significant differencecs (Tukey, $p < 0.05$).

### *3.4. Preparation of Biscuit with Selected Extract and Determination of Bioactivity*

After the results for the selected extract, it was decided to make a control biscuit and biscuits with different concentrations of extract (0.4, 0.9, 1.4%). See Figure 2B. The biscuit with the highest content of phenolic compounds compared to the control biscuit was the biscuit containing 1.4% extract (2.70 ± 0.19 mg eq GAE/g biscuit), without differences between the biscuits containing 0.4% and 0.9%. As for the antioxidant capacity, the biscuit with 1.4% of the extract showed the highest antioxidant power (4.50 ± 0.42 µMol eq TE/g biscuit) compared to the control biscuits and 0.4% and 0.9%. A relationship between the total amount of phenolic compounds and antioxidant capacity can be observed [27].

## 4. Conclusions

An extract with high antioxidant power and total phenol content was obtained using water as solvent. Four phenolic compounds were identified in the extract: gallic acid, catechin, rutin and chlorogenic acid. The extract subjected to the digestion process showed a bioaccessibility of 13% for total phenolic compounds. The use of the extract in the biscuit formulation showed an increase in total phenolic content and antioxidant capacity, making the extract a potentially functional ingredient for use in food processing. We need to conduct further studies on the encapsulation strategies.

**Author Contributions:** Conceptualization, A.M.; methodology, A.M. and A.I.; validation, A.M. and A.I.; formal analysis, M. R., M.B., A.I., and A.M.; investigation, M.R., A.I., M.I., and A.M.; resources, M.I. and A.M.; data curation, M.R. and M.B.; writing—original draft preparation, M.R. and M.I. and A.M.; writing—review and editing, M.R., M.I. and A.M; supervision, M.I., A.I., and A.M.; project administration, M.I. and A.M.; funding acquisition, M.I. and A.M. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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