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Separation conditions and evaluation of antioxidant properties of boldo (*Peumus boldus*) extracts

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Peumus boldus **is an endemic plant from the Republic of Chile which possesses important biological activities attributing at its antioxidant activity. Boldine had been reported as the main bioactive component of boldo. However, recently it has been shown the contribution of polyphenolic compounds in its bioactivity. Boldine, total polyphenolic content and antioxidant activity from *P. boldus* were recovered under several extraction conditions to obtain further information on the correlation between boldine and phenolic content with the potential antioxidant of *P. boldus*. Antioxidant potential was evaluated by scavenging effects of DPPH (2,2 diphenyl-1-picrylhydrazyl) radicals. The boldine and phenolic content were determined by high performance liquid chromatography analysis (HPLC) analysis and Folin-Ciocalteu colorimetric assay respectively. Our results showed that boldine yield increased as extraction time was raised from 6 to 24 h, in contrast total polyphenolic content decreased at this time. The maximum boldine content observed was 1.2% in stem at 75°C after 24 h of extraction. While the maximum total polyphenolic content observed was 4% in leaves at 70°C after 6 h of extraction. The optimal extraction conditions for the maximal antioxidant activity were using leaves at 50°C and 6 h extraction under heat-reflux. Low relationship was observed between boldine content and antioxidant activity. The results indicated that the antioxidant activity of natural phenolic compounds from *P. boldus* is highly influenced by the extaction conditions employed.**

Key words: Heat-reflux extraction, boldine, polyphenols, antioxidant activity.

INTRODUCTION

In recent years natural antioxidants have gained interest in food research as an alternative for substitution of antioxidants synthetic substances (Huang et al., 2005). This is mainly due to the fact that synthetic antioxidant have high manufacturing cost, some of them are toxic, causing pollution and sometimes presenting lower efficiency than naturals ones (Moure et al., 2001). Therefore natural antioxidants are taking importance due to their beneficial properties in human health as decreasing and preventing some pathologies, they are obtained from a renewable source and are very important; they are obtained at low cost using efficient procedures. Besides, in food industries, antioxidants are used to prevent fats and oils oxidation which provoke undesirable smell and flavors and in extremes cases formed toxic compounds (Moure et al., 2001; Oliveira et al., 2008). By the reasons mentioned above, the study of natural antioxidants obtained from vegetables sources as alternative to

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replace synthetic antioxidants is nowadays an important area of research.

Peumus boldus (Monimiaceae) or commonly, knowing like boldo, is an endemic plant from the Republic of Chile which has been used in the folk medicine due to various biological effects like anti-inflammatory, antipyretic, hepatoprotective, anti-carcinogenic and antioxidant (Backhouse et al., 1994; Rodrigues et al., 2002; De la Fuente et al., 2005; Del Valle et al., 2005; Gerhardt et al., 2009). Most of these biological effects could be attributed at its strong ability of scavenger free radicals (Del Valle et al., 2004; Srivastava et al., 2011).

Boldine (2,9-Dihydroxy-1,10-dimethoxyaporphine) is the most abundant alkaloid present in boldo extracts and it is considerate as the main natural antioxidant at low concentrations (Srivastava et al., 2011). Despite of this, different authors have suggested that boldine may not be the unique compound with great antioxidant activity in boldo extracts. Because leaf extracts are more used in nature medicine despite bark extracts contain higher levels of boldine than those present in leaves (Del Valle et al., 2004). Together, Simirgiotis and Schmeda-Hirschmann (2010) suggested that the strong free-radical activity is attributed mainly to polyphenols compounds such as catechin and flavonoids rather than to boldine due to the relative concentration present in the boldo extracts. Therefore the aim of the present study was: to determine time and temperature effect on extraction process of boldine and total polyphenols content from *P. boldus* and their relationship with free radical scavenging activities.

MATERIALS AND METHODS

Recollection and preparation of plant material

Dried leaves and stems of *P. boldus* was obtained from a local market at the Mexico City on January 2008. However, plant sample was collected from Nogales, Sonora, Mexico on September 2007 (trader's information). The vegetal samples were maintained in black plastic bag and translated to the Food Research Department, School of Chemical Sciences, Universidad Autonoma de Coahuila at Saltillo City, Coahuila, Mexico. Leaves were separated from stems and both tissue samples were ground separately to a 6 mm particle size. The vegetal material was dried on a stove (NAPCO, model 322) at 40°C for 48 h to have a uniform weight (Del Valle et al., 2004; Coquelet et al., 2007).

Conditions for boldine extraction

In this study, the conventional solid–liquid extraction was used in order to observe the suitable conditions for boldine and phenolic compounds recovery in two steps. In the first step, a dried fine powder sample (10 g) of each different tissues (leaves, stems and a mixture (1:1) of both tissues) were placed in an Erlenmeyer flask respectively covered with aluminum foil to avoid light exposure, then 80 ml of 70% ethanol were added, because is known that the boldine and phenolic compounds are soluble in polar solvents like water, hydro-alcoholic solutions and acid solutions (Palma et al., 2002). The flasks with the mixture, leaves and stem samples were refluxed at three temperatures 30, 50 and 70°C and monitored during the extraction at 2, 4 and 6 h in Eppendorf-tube of 2 mL covered with aluminum.

In the second step, the extraction consisted in increase temperature and prolongs the extraction time. In this case, the conditions were 65, 70 and 75°C monitored at 6, 12 and 24 h. Extracts from the two steps were stored at 4°C in dark conditions before the boldine quantification using high performance liquid chromatography.

High performance liquid chromatography analysis

Boldine quantification of each extracts was carried out using high performance liquid chromatography analysis (HPLC) in reverse phase. A Varian Pro-Star 330 photodiode array (PDA) detector with detection at 280 nm was used. Samples fractionation was performed on an Eclipse XDB- C18 column (5 µm, 4.6 × 15 mm) under as follow: it was used an isocratic regime and a mobile phase consisting of methanol and water in a relation 70:30 and applied at a flow rate 0.3 mL/min, with a sample injection volume of 5 µL (sample previously filtered through a 0.45 µm nylon membrane and diluted 1:10). In this method a standard solution of different concentrations (from 0 to 200 mgL^{-1}) was prepared by dissolving boldine ((s)-2-9-dihidroxi-1, 10-dimethoxiaporfine, Sigma Aldrich) in ethanol (70%) and it was used as standard curve for calibration and quantification of extracts by linear correlation.

Determination of total phenolic content

Total phenols content was obtained from the amount of hydrolysable and condensed polyphenols. For this quantification were selected the extracts that presented the highest levels of boldine according to the extraction time in each step. The quantification of hydrolysable polyphenols (Makkar, 1999) was evaluated as gallic acid equivalents where a reference curve was performed from 0 to 200 mg L^{-1}. First, the sample was diluted (1:50) in test tube covered with aluminum. Briefly, 400 µL of the sample were placed in other test tube for reaction, then were added 400 µL of Folin-Ciocalteu (Sigma-Aldrich) reagent, shaken and left for 5 min. Then 400 µL of Na_2CO_3 (0.01 M) were added and shaken and left for 5 min again. Finally, the solution was diluted with 2.5 mL of distilled water and absorbance was read at 725 nm. The hydrolysable polyphenols concentration in each extracts was obtained according to the reference curve by linear correlation.

Condensed polyphenols content was determined from the same diluted sample. Samples 0.5 mL was mixed with 3 ml of HCl/butanol (1:9) and 0.1 mL of ferric reagent and the test tube with the mixture was closed and heated for 1 h at 100°C in a water bath. After that, it was cooled and absorbance was read at 460 nm. A reference curve of catechin was done (0 to 500 mgL^{-1}) and condensed polyphenols content was expressed as catechin equivalents obtained by linear correlation.

Antioxidant activity assay using 2,2 diphenyl-1-picrylhydrazyl (DPPH)

Antioxidant activity was determined for those extracts that presented the highest levels of boldine at each extraction time in each step. In this case, antioxidant capacity of boldine extracts was evaluated using DPPH free radical-scavenging activity, following the method reported by Brand-Williams et al. (1995), which is

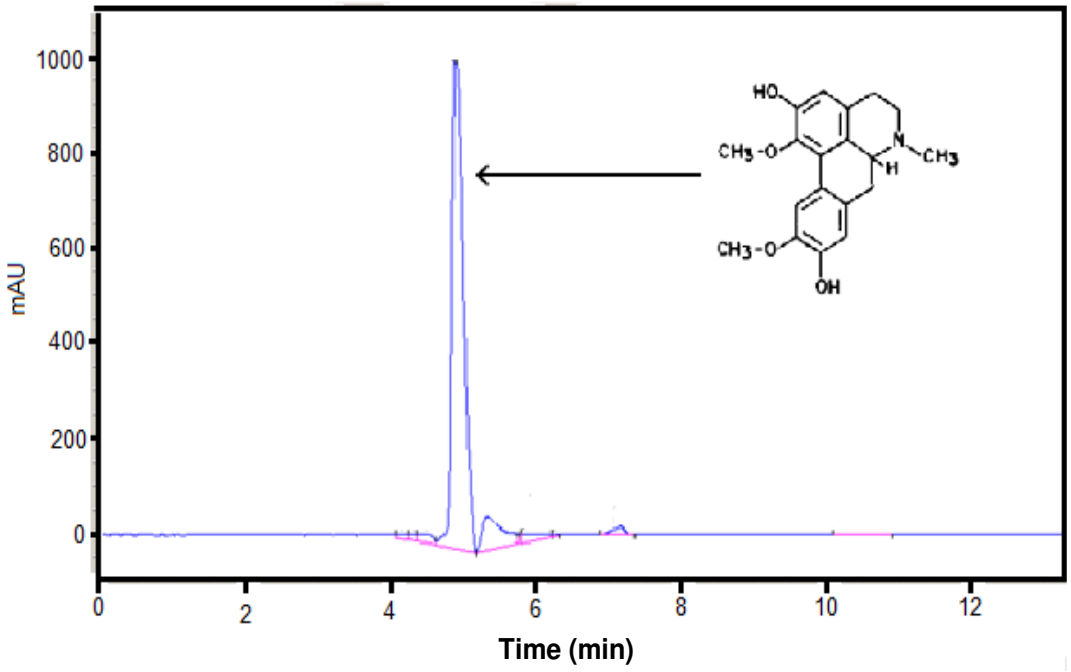

Figure 1. HPLC chromatogram and chemical structure of alkaloid boldine reagent.

described briefly: a solution of 25 mgL^{-1} of DPPH radical was prepared in methanol and stored in cooler and dark conditions. Then, 2.9 mL of solution of DPPH free radical were placed and was added 0.1 mL of the diluted extract sample (1:400), free radical-scavenging activity reaction was monitored reading the absorbance in an Uv-Vis spectrophotometer at 515 nm. The radical-scavenging activities of extracts samples were expressed as antioxidant activity and were calculated according to the formula:

$$\text{Antioxidant activity } (\%) = \frac{(A_C - A_S)}{A_C} * 100$$

Where: A*c* is control absorbance and A*s* is absorbance of sample.

Characterization of boldine extract

Once selected the extract with the highest levels of antioxidant activities, it was obtained in higher amounts for quantification of boldine content using HPLC, total polyphenols amount and its percentage of DPPH free radical scavenging were also determined, then they were compared against commercial boldine (Sigma-Aldrich, 99% pure). Solvent was separated from the obtained extract using a rotary evaporator (50°C).

Statistical analysis

The experiments in the two steps were performed under a completely randomized design with a factorial arrangement 3^3 with three replications. When it was needed, mean treatment comparison were done estimating standard deviation. Data were analyzed using the SAS V 9.0 software.

RESULTS AND DISCUSSIONS

Suitable conditions of bioactive compounds extraction

The extract of boldo leaves contains a wide range of phytochemical such as essential oils, isoprenoids, flavonoids, various alkaloids and other compounds (Srivastava et al., 2011). Boldine is the main alkaloid present in boldo. The chemical structure and the HPLC chromatogram of boldine are shown in Figure 1. A representative separation of boldine was achieved within 5 min, which was helpful for its fast quantification. In the first extraction step were evaluated three factors (tissue, temperature and time) on boldine content extracted. The results shown it were necessary 6 h of extraction to obtain the maximum boldine content (data no shown); therefore, this time was selected as an appropriate extraction time in the first step. The effect of different levels of temperature on boldine extraction is observed in Figure 2a. In general, extraction efficiency of boldine increased as temperature does. The result suggested that 70°C is the most adequate temperature for boldine extraction. In addition, Figure 2a shows the extracted boldine content from leaves, stems, and a mixture (leaves and stem) at 6 h. The maximum content of boldine found in leaves, stems, and a mixture of both tissues at 70°C was 0.47, 0.60 and 0.53 mg of boldine/g of dry plant material respectively. Clearly it was observed

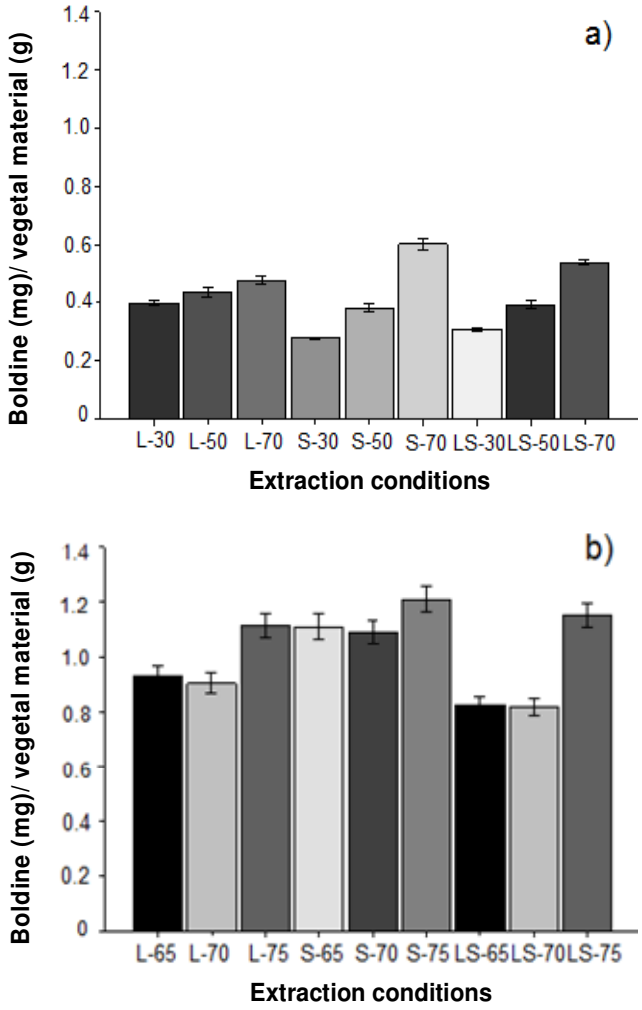

Figure 2. Effect of tissue, temperature and time on boldine extraction at 6 h (a) and 24 h (b). Leaves (L), Stem (S), Leaves and stem (LS) and different temperatures (30, 50, 65, 70 and 75°C).

that at this temperature, stems have greater amount of boldine than leaves and mixture of tissues. The better extraction conditions to obtain the maximum boldine yield in the first step were determined as 70°C and 6 h of extraction using stems tissue.

The second step for boldine extraction was carried out in order to enhance boldine yields. In this step, it was observed that boldine increased as extraction time does from 6 to 24 h (data not shown). Figure 2b shows boldine content at different high temperatures in all plant tissue evaluated. It is clear that boldine content reached the highest values at 75°C, in the all evaluated tissues. In the same way, the maximum boldine yield in the second step was observed in the stem tissue sample as in the first extraction step. The maximum yield of boldine in the first step (0.60 mg boldine/g of dry plant material) was duplicated under the conditions of the second step (1.2 mg boldine/g of dry plant material).

Total phenolic content

The values of total phenolic content (hydrolysable polyphenols and condensed polyphenols) in the extracts with maximum boldine content at 6 and 24 h are shown in Figure 3. In contrast with boldine yield, total phenolic content in boldo leaves was affected negatively with larger extraction times. Total phenolic content in boldo extracts decrease in the most of cases evaluated at 24 h of extraction. Then, the maximum phenolic content (41 mg/g of dry material) was observed in boldo leaves at 6 h and 70°C.

2,2 Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

Figure 4 shows the DPPH scavenging activities of boldo extracts obtained using different (tissue, temperature and time) extraction factors. Temperature factor seems have not significant effect on the DPPH scavenging activity (Figure 4a). In contrast, tissue and extraction time had significant effect (Figure 4). In general, the extracts obtained at 24 h of extraction showed lowest antioxidant activity compared with those obtained at 6 h. It is important to note, the maximum content of boldine was observed in extracts obtained in larger times (24 h) and the maximum phenolic content and maximum antioxidant activity were observed in extracts obtained in short times (6 h). Besides it is known that larger exposure time affects some polyphenolic compound (Ballard et al., 2010).

Characterization of the boldo extract

The boldo extract with the maximum antioxidant activity (leaves, 6 h and 50°C) was characterized respect to boldine content, polyphenolic amount and antioxidant activity (Table 1). The boldine amount was 360.99 mg/L and the total polyphenolic content was 21.17 g/L. It is important to note that 99.57 % of the total polyphenolic content was polyphenols from the condensed group. Finally, the antioxidant activity of the boldo extract was quantified and compared with the antioxidant activity of boldine reagent. Figure 5 shows the increase of inhibition percent respect to the time for boldo extract and boldine extract. The maximum antioxidant activity was achieved at 30 min with a percent of inhibition of 92.66 % which was higher than the boldine reagent (74.47 %). Thus, we can say that the polyphenolic compounds potentiate the total antioxidant activity of boldo extracts together to the

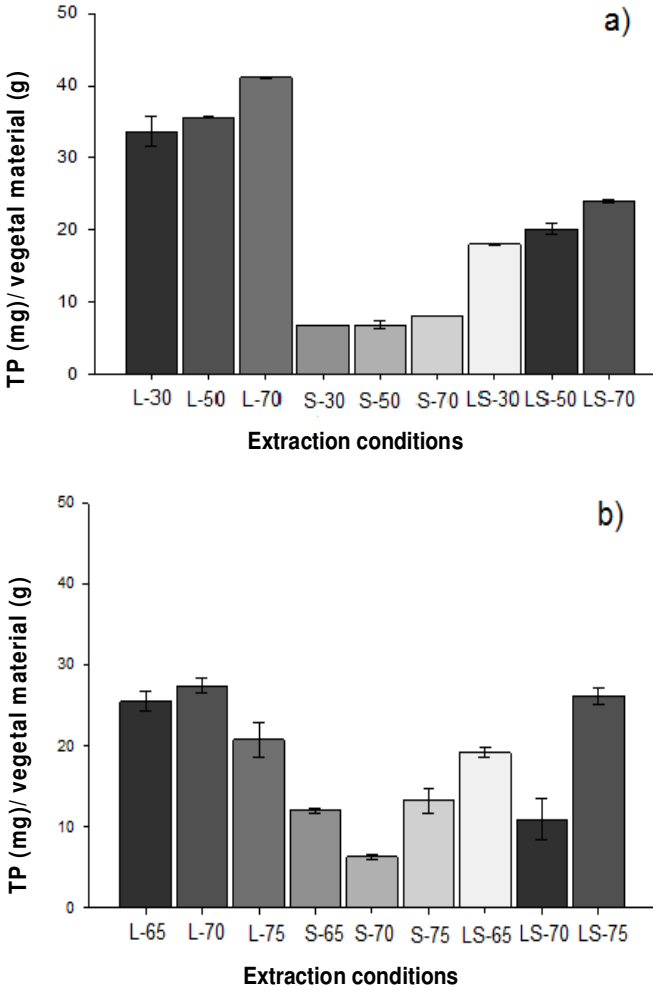

Figure 3. Effect of tissue, temperature and time on total polyphenols (TP) extraction at 6 h (a) and 24 h (b). Leaves (L), Stem (S), Leaves and stem (LS) and different temperatures (30, 50, 65, 70 and 75°C).

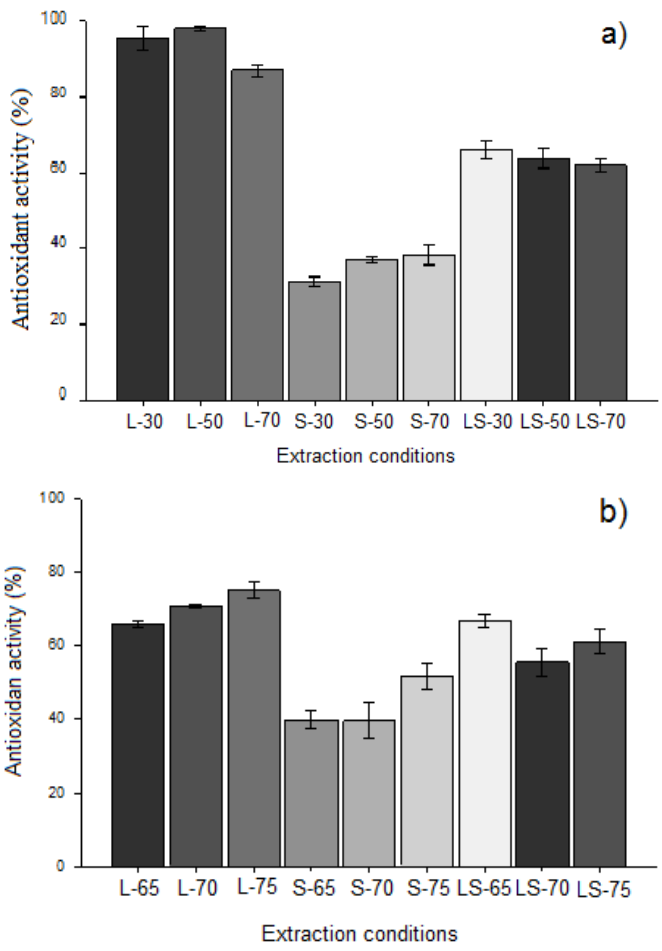

Figure 4. Effect of tissue, temperature and time on the antioxidant activity (%) by DPPH free radical-scavenging method at 6 h (a) and 24 h (b) of extraction. Leaves (L), Stem (S), Leaves and stem (LS) and different temperatures (30, 50, 65, 70 and 75°C).

boldine compound.

In the first extraction step it was observed that the time and temperature were the most important factors during boldine extraction process. The highest temperature evaluated resulted in the increase of boldine extraction. This may be attributing to increase of temperature favored extraction by enhancing both diffusion phenomenon and solute solubility. However, degradation of compounds of interest can be carried out at high temperature (Ghafoor et al., 2009). In addition this range of temperature and prolong extraction times play an importance role in the stability of plant structural components such as proteins (Francisco, 1999) polysaccharides and lignin. Then, these modifications can be reversible and irreversible depending of specific composition structural in each plant tissue. In several cases, irreversible modifications could produce cellular disruption and explain the release of the intercellular and linked target compounds increasing the yield extraction. Similarly to boldine yields extracted in our experiments, Quezada et al. (2004) quantified boldine content in boldo leaves with yields of 1.4 mg boldine/g of dry plant material and O'Brien et al. (2006) reported yields of 1.2 mg boldine/g of dry plant material. Various factors as seasonal, stresses and plant tissue have an important effect on alkaloid levels in boldo barks (Del Valle et al., 2004) and the entire plant.

Boldo leaves extract had significantly highest antioxidant activity, followed by the combination of both tissues. Moreover, the extracts obtained at long time extraction showed lowest antioxidant activity compared with shorter one. It is important to note that the maximum boldine yield was observed from stem tissue extracts (Figure 2b) and the maximum polyphenolic content was obtained in leaves tissue extract (Figure 3a). In addition,

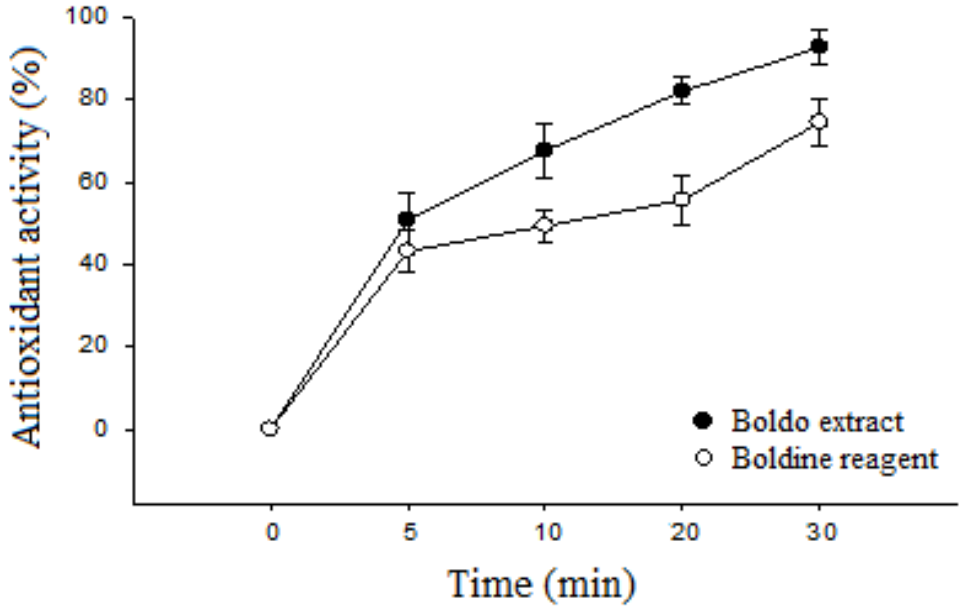

Figure 5. Comparative DPPH free radical-scavenging activity of the boldo extract and boldine reagent.

Table 1. Boldine, polyphenolic content and antioxidant activity of the boldo extract.

Boldo extract	Boldine content (g/L)	Hydrolysable polyphenols content (g/L)	Condensed polyphenols content (g/L)	Total polyphenolic content (g/L)	Antioxidant activity (%)
Leaves, 6 h and 50°C	0.36	0.09	21.08	21.17	92.66

the maximum content of boldine was also observed in extracts obtained in long times (24 h) and the maximum phenolic content was observed in extracts obtained in short times (6 h). It is known that larger exposure time affects some polyphenolic compound (Ballard et al., 2010). Therefore, these results could suggest that the antioxidant activity of boldo is more related to other compounds like these polyphenolic compounds instead of boldine. We findings are agree with Del Valle et al. (2004), and Simirgiotis and Schmeda-Hirschmann (2010) who mentioned that boldine have a low contribution at antioxidant activity in boldo extracts attributing the major antioxidant activity to phenolic compounds presents. Overall, we can suggest that there is higher relationship between the polyphenolic compounds with antioxidant activity instead of boldine. However, is important mention that is necessary a deep study to be sure in the total contribution of each purified compound. It is important to note that most of the total polyphenolic content was polyphenols from the condensed group. Inside this group are potent antioxidants such as catechin and flavonoid, which were reported in boldo extract by Simirgiotis and Schmeda-Hirschmann (2010).

Conclusion

The results of this study showed that several experimental parameters such plant tissue, temperature and extraction time had a great influence on boldine and polyphenolic compounds yields from *P. boldus*. These results are helpful for the future experiment where extraction conditions need to be optimized for extraction of boldine and polyphenolic compounds. However, further studies will be developed to characterize, identify and isolate the specific compounds responsible of the maximum antioxidant activity of *P. boldus* extracts. *P. boldus* extracts could be used as natural potential antioxidant, especially if leaves tissue is used in the extraction.

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