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Phytochemical variation of wild and farmed populations of boldo (*Peumus boldus* Molina)

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ABSTRACT

The phytochemical profile of six wild and one cultivated *Peumus boldus* (boldo) populations from different regions of north-central, central and south-central Chile was studied. In leaves, wood, and bark, alkaloids and phenolics were analyzed by UHPLC-MS-MS and leaf essential oils by GC-MS. In each population, compounds were found to exhibit high variability, but important differences were recorded at the population level. The north-central wild populations showed higher concentrations of alkaloids and polyphenols in leaves and alkaloids in the bark compared to more southern populations. Saplings farmed under different shade conditions contained higher species-characteristic leaf polyphenolic concentrations with increasing light while most alkaloids increased with the shade. When analyzed the following year, higenamine, boldine, isocorydine and *N*-methyllaurotetanine increased. The principal components of the leaf essential oils from the wild populations were p-cymene, ascaridole and 1,8-cineole, while in the farmed trees ascaridole was replaced by its precursor α-terpinene as the second most abundant constituent. Although multiple factors may affect the concentration of secondary metabolites and geographic provenances with its attending differences in sunlight and rainfall has been suggested as one of these, the present work shows that latitude by itself cannot explain differences that have a clear impact on quality from the medicinal standpoint.

1. Introduction

The boldo, *Peumus boldus* Molina (Monimiaceae), is a representative endemic tree of the Chilean sclerophyllous forest. Its geographical range in central Chile covers more than seven degrees of latitude, in a strip about 1100 km long with an approximate area of 4486 km^2 (Benedetti Ruiz et al., 2009; Benedetti and Barros, 2011). In central Chile, the biomasses of *P. boldus* and *Quillaja saponaria* Molina (Quillajaceae) have been for decades major profitable resources and some of the few limited economic opportunities for owners of native forest plots (Aguirre and Infante, 1988; Benedetti Ruiz et al., 2009).

The recorded use of its leaves by humans goes back about 14,000 years (Dillehay et al., 2008; Pino and Dillehay, 2023), and these are widely appreciated in Chile and abroad as a plant drug, mainly for digestive complaints (European Medicines Agency, 2016; European Pharmacopoeia, 2013; Fundación Farmacopea Chilena and Universidad de Valparaíso, 2016; Speisky and Cassels, 1994). A boldo leaf infusion is often drunk after meals in much of South America, as it is believed to aid digestion and is supposed to increase the flow of bile (Michetti et al., 2019; Rezende et al., 2017; Speisky and Cassels, 1994). Currently, each year, 2000 metric tons of boldo leaves are shipped as a commodity (Benedetti Ruiz et al., 2009). For these reasons, this species is viewed by

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Chilean researchers as one of the five most important native trees providing non-timber forest products (NTFP) (Valdebenito et al., 2015).

The wide latitudinal range of *P. boldus* implies differences in both climate and soil that make it a very hardy and plastic species growing in very diverse environmental conditions (Donoso et al., 2011). Thus, historical annual rainfall at the northern limit of its range is 130 mm/year with an 8–9-month dry season, while near its southern limit, rainfall is more than 2000 mm/year with no dry season. Effects of environmental features on secondary metabolite production in medicinal plants have been reviewed recently (Li et al., 2020; Pant et al., 2021), but not along a geographic continuum for a species growing wild as in our case.

The exploitation of this resource has made a strong impact on the structure of vegetation formations and on the architecture of each tree (Benedetti and Barros, 2011). Different sources indicate that technical knowledge needs to be stepped up and disseminated to ensure that official forest management guidelines are followed and are the most adequate (Benedetti Ruiz et al., 2009; Donoso et al., 2015). The pressure on wild populations has led to the widespread idea that boldo farming should be encouraged (Benedetti Ruiz et al., 2009; Vogel et al., 2011). Small trials are in progress (Doll et al., 2005; Peña-Rojas et al., 2018; Vogel et al., 2011). Nevertheless, little information is available regarding the effect of insolation on the quality of farmed products. Only one trial has analyzed the effect of shade on the yield of essential oil and percentage of alkaloids in boldo, without finding any differences (Vogel et al., 2011). The effect of light intensity on the production of isoquinoline alkaloids has been studied in a temperate species (Kong et al., 2016), and our own work on *Cryptocarya alba*, another Mediterranean-type climate sclerophyllous tree. However, we have found differences attributed to the effect of shade (40 % v/s 80 %) in some alkaloids and polyphenols in *Cryptocarya alba* (Mol.) Looser, another sclerophyllous species (Giordano et al., 2019).

From our perspective, it is of the utmost importance to face this problem immediately. 100 % of the current harvest of this species is based on wild populations, where most of the individuals are hundreds of years old. At present these trees easily throw up new suckers after harvesting, but there is no certainty that this will continue to be the case because of how the landscape is being transformed, advanced age of most of the trees, and due to increasing aridity owing to climate change. There are proven strategies to improve the value of these products, such as certification and fair trade, focused on partnership and good practices (Duchelle et al., 2014). Fluctuations in the phytochemical profile can be expected to affect boldo's medicinal properties (Gobbo-Neto et al., 2017) and in this sense, our work can be applied to the design of quality standards, secondary metabolite oriented forestry and phytochemical standardization (Joshi, 2012).

In regulatory matters it has been stated that the leaves contain 1.2 % tannins and flavonoid phenolic compounds, plus a minimum of 0.1 % of total alkaloids expressed as boldine which is assumed to be the main alkaloid, and 20–40 mL/kg of essential oil, mainly composed of p-cymene, cineole, and ascaridole (Fundación Farmacopea Chilena and Universidad de Valparaíso, 2016). The presence of about thirty different compounds has been documented in the leaves using a UPLC-QTOF-MS profiling method (Steenkamp et al., 2018). The major alkaloids, terpenes, and phenolic compounds of *P. boldus*, many with valuable pharmacologic activities, are sufficiently known (Bakiri et al., 2017; Fuentes-Barros et al., 2018; Kuhn et al., 2019; Torres-Vega et al., 2020).

Among the factors that have been studied for boldo leaves, their content of the alkaloid boldine is known to vary widely within and among populations, apparently trending along a north-south axis (Fuentes-Barros et al., 2014). Higher alkaloid concentrations towards the equator seem to be a common phenomenon in many species and it has been suggested to occur in several alkaloid-bearing plants in Chile (Niemeyer, 2014). Differences throughout the country have also been detected for boldo leaf essential oil (EO) (Vogel et al., 1999).

In our previous paper (Fuentes-Barros et al., 2018), we analyzed the alkaloids present in the leaves of 130 individual trees growing wild in Central Chile. Roughly, in any single population, we found interannual variations, a weak sex-related effect for this dioecious species, and higher alkaloid concentrations in mature leaves than in new ones. In the harvested trees, higenamine (norcoclaurine), reticuline, and laurotetanine increased, isocorydine decreased, and the other alkaloids were the same as in those not harvested in the same area. On analyzing new and old suckers of individual trees, the leaves from new ones are significantly richer in most of the alkaloids. Also, the alkaloid content in the harvested population was lower than that in old trees (DBH >40 cm) from a nearby population and in farmed trees two years after planting. On this basis, we designed the present project to compare the alkaloid, polyphenol, and EO contents in six populations growing from about 32–39° South, sampling individuals from unharvested wild populations, and also to evaluate the effect of shading on farmed trees. This is the first comprehensive quantification of the boldo alkaloids and polyphenols in wild populations considering leaves, wood, and bark, and the leaf essential oils, and in the leaves of farmed trees. We also compared our new essential oil results with those for wild harvested and unharvested trees from our previous work.

2. Materials and methods

2.1. Collection sites

Six provenances of *P. boldus* in north-central, central and south-central Chile were selected. 25 individuals were chosen from each of them and evaluated for height, stump diameter, foliage density, number and diameter of suckers and overall health (Instituto Forestal, 2018). Of these, 10 individuals were chosen for this work from each provenance (Table 1).

Boldo suckers were sampled, and bark and wood were separated. Samples were also taken from saplings growing in an experimental farm in San Bernardo (Central Valley) next to the city of Santiago, Chile (33°39'S and 70°43'W, 550 masl). The farmed plants were from a nursery in the transitional region between Mediterranean and temperate climates, whose seeds had been collected in the surrounding area (37°28′S and 72°2 W). Once planted, they were grown under artificial irrigation, never treated with fertilizers or pesticides, and under different shading conditions. Raschel 80 % shade netting was placed over the last 12.5 m at the ends of the 50 × 12 m planted area, while the 40 % shade netting was placed over the remaining central 25 m. Of the latter, 3.5-year-old individuals ($n = 50$), half of them grown under 80 % shade ($n = 25$) and half under 40 % shade ($n = 25$), were used to extract their leaf EOs and the same saplings were used to analyze the alkaloid content the following year. EO analyses were also done comparing trees that had been harvested regularly every five years under an approved management plan ($n = 30$) with trees that had not been harvested for decades (protection zone) ($n = 20$), all growing near María Pinto (Fig. 1) and (Fuentes-Barros et al., 2018).

2.2. Chemicals and reference standards

All solvents used were of analytical grade or double-distilled. Water was of ultrapure grade. Folin-Ciocalteu reagent, sodium carbonate, sodium acetate, sodium sulfate, potassium persulfate, acetic acid, ferric chloride, and aluminum chloride hexahydrate were purchased from Merck. The purity of all standards was over 98 % by HPLC. Gallic acid, quercetin, chlorogenic acid, ferulic acid, caffeic acid, myricetin, syringic acid, vanillic acid, apigenin, pinocembrin, kaempferol, and *p*-coumaric acid, linalool, limonene, terpinen-4-ol, γ-cimene and eucalyptol (1,8-cineole) were purchased from Sigma-Aldrich. α-Pinene, β-pinene, sabinene, catechin, epicatechin, rutin, isorhamnetin, quercitrin, cryptochlorogenic acid, isocorydine HCl, and procyanidins B1, B2, and C1 were purchased from Phytolab. The alkaloids were either isolated, prepared synthetically or obtained as gifts as reported recently by us

Table 1
Location of studied populations.

Region	Sector	Altitude masl	Geographic coordinates	Topography	Climate
Coquimbo	Los Vilos	173	31°54'S 71°30'W	Ocean coast	Semiarid
Valparaíso	Nogales	376	32°43'S 71°12'W	Interior valley	Mediterranean
Metropolitana	María Pinto	230–250	33°26S 71°18W	Central valley	Mediterranean
O'Higgins	Doñihue	433	34°13'S 70°57'W	Central valley	Mediterranean
Maule	Rauco	200	34°55'S 71°18'W	Central valley	Mediterranean
Ñuble	San Fabián de Alico	552	36°33'S 71°33'W	Andean foothills	Temperate
Araucanía	Pucón	225	39°16'S 71°58'W	Andean foothills	Temperate

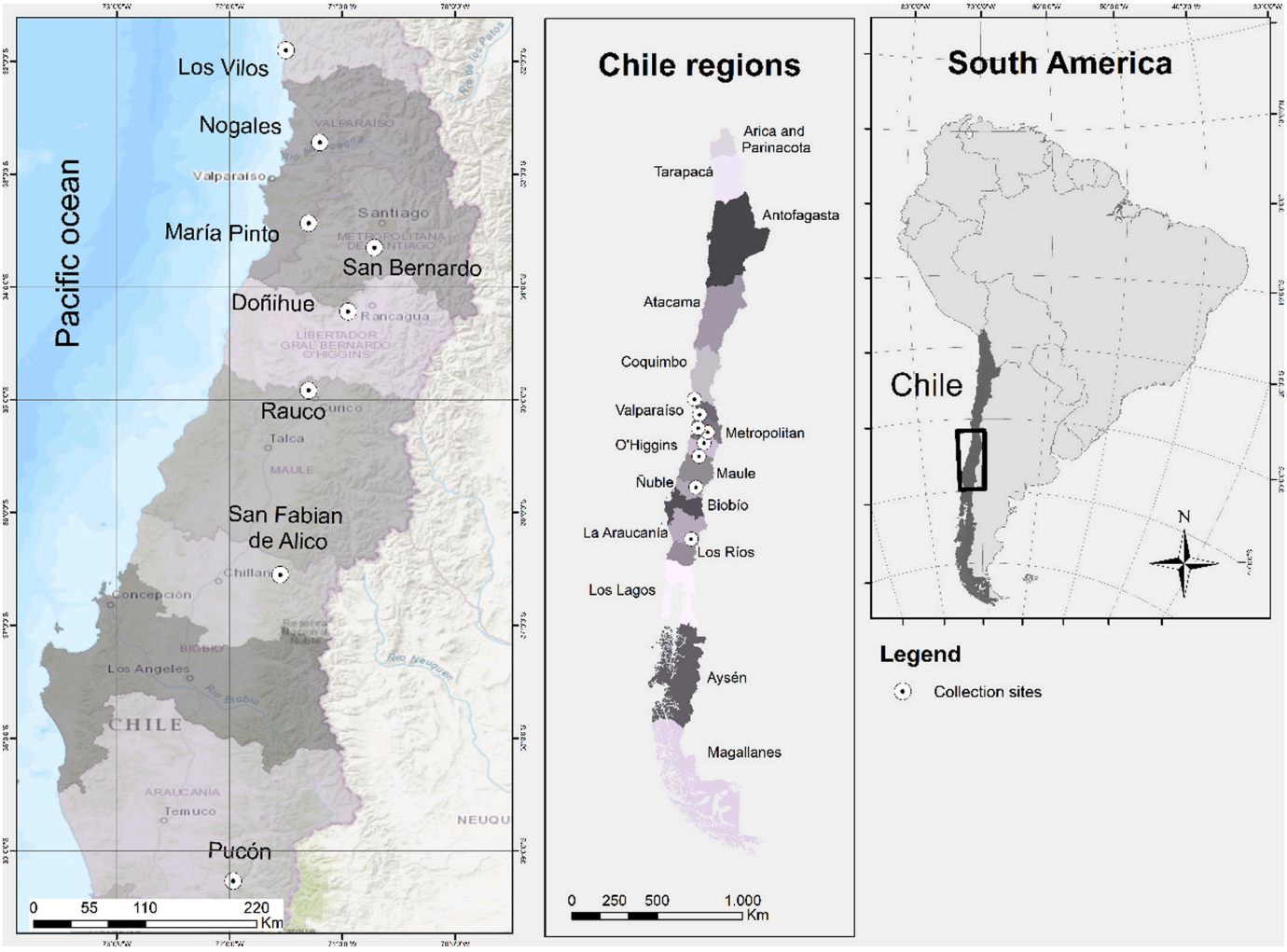

Fig. 1. Collection sites in Chile.

(Fuentes-Barros et al., 2018).

2.3. Preparation of the samples

2.3.1. Preparation of methanolic extract

All samples were dried in an oven at 40 °C for 72 h after a few days of storage in a room at about 22 °C with low humidity, and then pulverized in a table grinder and passed through a screen (<2 mm). Aliquots weighing 1 ± 0.01 g were suspended in methanol (100 mL), stirred for 2 h at 60 °C, and filtered through Whatman No. 1 paper. The solid collected was subjected to the same procedure 4 times. The five extracts were combined and concentrated in a rotary evaporator at 40 °C under reduced pressure and stored until use in amber vials. For analysis, the residues were resuspended in methanol (20 mL).

2.3.2. Extraction of essential oils

The collected leaves were placed in a cooler to keep them fresh during transfer to the laboratory, and then kept refrigerated at −20 °C until use. The EOs were extracted using a steam hydro-distillation apparatus with a 2 L water capacity. For each individual tree, 40 ± 0.05 g of fresh leaves were used, with an extraction time of 4 h. The EO was separated by decantation, dried with anhydrous sodium sulfate, and immediately stored at −20 °C in an amber vial.

2.4. Quantification of total polyphenol content (TPC) and total flavonoid content (TFC)

The polyphenol content was quantified colorimetrically (Singleton and Rossi, 1965). 50 μL of the sample, 250 μL of Folin-Ciocalteu reagent (2.0 M) and 3 mL of distilled water were mixed, and after five minutes

Na_2CO_3 solution (750 µL, 20 % w/w) and 950 µL of distilled water were added. This mixture was vortexed, incubated at room temperature for 40 min, and its absorbance recorded at 765 nm. The calibration curve was built using gallic acid (Table S1). The results were expressed as mg of gallic acid equivalent (GAE)/g DW (dry weight). The flavonoid content was determined following a recent literature method (Chang et al., 2002). 300 µL of sample, 2.5 mL of distilled water, sodium acetate solution (1 M, 100 µL), and aluminum chloride hexahydrate solution (10 % w/v, 100 µL) were vortexed, incubated at room temperature for 30 min, and the absorbance recorded at 435 nm. The calibration curve was built using quercetin (Table S1). The results obtained were expressed as mg of quercetin equivalent (QE)/g DW.

2.5. Liquid chromatography and mass spectrometry

All the samples were injected into an ultra-high pressure liquid chromatograph (Eksigent model EkspertUltraLC 100-XL) coupled to a triple quadrupole mass spectrometer in the electrospray mode (ESI) (ABSciex Triple Quad 4500). A Phenomenex Synergi™ Fusion-RP 80 Å (50 mm × 2.0 mm, 4 µm) column was employed and the injection volume was 10 µL. The LC-MS/MS system was controlled by Analyst 1.6.2 and the data processed by Multiquant 3.0.

2.5.1. Quantification of alkaloids

The mobile phase was aqueous formic acid 0.1 % v/v (eluent A) and acetonitrile (eluent B), at a flow rate of 0.3 mL/min. The gradient program (Fuentes-Barros et al., 2018) was as follows: (time, min/%B) 3/3 %, 13/15 %, 16/20 %, 17/3 %, 18/3 %. The mass spectrometer parameters were: GS1 nitrogen, (40 psi); GS2 nitrogen, (50 psi); IS, 3500 volts, TEMP, 650 °C; CURT nitrogen (25 psi). Calibration curves were built with concentrations in the range 0.1–0.8 µg/mL, the equations of each compound are presented in Table S1.

2.5.2. Quantification of polyphenols

The mobile phase was aqueous formic acid 0.1 % v/v (eluent A) and acetonitrile (eluent B) at a flow rate of 0.5 mL/min. The gradient program (Pastene et al., 2014) was as follows: (time, min/%B) 1/5 %, 10/40 %, 11/100 %, 13/5 %, 14/5 %. The mass spectrometer parameters were: GS1 nitrogen, (50 psi); GS2 nitrogen, (50 psi); IS, 4500 volts, TEMP, 650 °C; CURT nitrogen (30 psi). Calibration curves were built with concentrations in the range 0.1–0.8 µg /mL, the equations of each compound are presented in Table S1. Procyanidins B represents the sum of procyanidins B1 and B2.

2.6. Essential oil chromatographic analysis

GC-MS analysis was performed with a Shimadzu model GCMSQP 2010 Ultra gas chromatograph (Shimadzu, Kyoto, Japan), equipped with an Rtx-5MS Crossbond 5 % diphenyl - 95 % dimethyl polysiloxane (Restek, Bellefonte, PA, USA) capillary GC column (30 m length, 0.25 mm I.D., 0.25 mm film thickness). The GC was operated in the splitless injection mode and the injection volume was 5 µL. The column temperature was held at 35 °C for 5 min, raised at 10 °C/min to 200 °C, and maintained for 10 min at 200 °C. The carrier gas was helium at a flow rate of 1.3 mL/min. The mass spectrometer was used in the electron impact ionization mode (70 eV) with an emission current of 250 mA, scan time, 0.3 s, and acquisition mass range, 50–500 dalton. The temperatures of the injection port, ion source, and transfer line were 250, 250, and 250 °C, respectively. The instrument was operated in the scan mode. The identification of compounds in the chromatographic profiles was achieved by i) comparison of mass spectra with those in the NIST14 library database; and ii) comparison of Kováts indexes (KI) with those reported in the literature, or with those of available standards. The quantification of compounds was carried out using peak area integration.

2.7. Statistical analysis

The distribution of each variable was examined using the Anderson Darling tests for normal distribution and Bartlett's test of homoscedasticity. When the distribution was normal, a general linear model was used to analyze the effect and then differences between groups were discerned using Tukey's a posteriori method. The variables that did not behave normally in a transformation were analyzed using the Kruskal Wallis nonparametric test. The t-test was used for paired groups when their distribution was normal, and the Mann-Whitney *U* test when it was not. The correlation coefficients used were Pearson's for the variables with normal distributions, and Spearman's Rho when normality was absent. Minitab® 18 and GraphPad Prism 8 statistical packages were used.

3. Results

Periodic harvesting affects the alkaloid and polyphenol content (Fuentes-Barros et al., 2018) and Fig. S1). The age of the suckers has a more marked effect on alkaloids, while sex has little impact on alkaloid and polyphenol content (Fuentes-Barros et al., 2018) and Fig. S2). Consequently, in the present work, only trees that had not been harvested for many years were considered, regardless of sex.

3.1. Phenolic compounds

The most abundant phenolics found in the leaves were catechin, epicatechin, and procyanidins. Some of these, particularly catechin, epicatechin, and rutin, showed important differences at the population level, decreasing towards the cloudier, cooler, more humid south, while the concentration of others such as quercitrin did not show any clear pattern (Fig. 2). Also shown are the total polyphenol and flavonoid contents of leaves of six populations of *P. boldus* (Fig. S3), with the northernmost populations having the highest content.

The compounds with the highest concentrations were procyanidins, catechin, and epicatechin in both bark and wood, with generally higher concentrations of polyphenols and flavonoids in the bark (Figs. 3 and 4). Interestingly, in the bark, the northernmost and southernmost populations presented the highest values, while the lowest occurred in the O'Higgins Region (Fig. 3). The results of the flavonoid and total polyphenol content of wood and bark of the six populations of *P. boldus* are also presented (Fig. S4).

No clear pattern was seen in the wood phenolics. However, the southernmost population presented the highest values, while the O'Higgins population again showed the lowest values, where no procyanidin-B was detected (Fig. 4).

Regarding the farmed individuals, those grown under 80 % shade had larger leaves than those with 40 % shade, but there were no

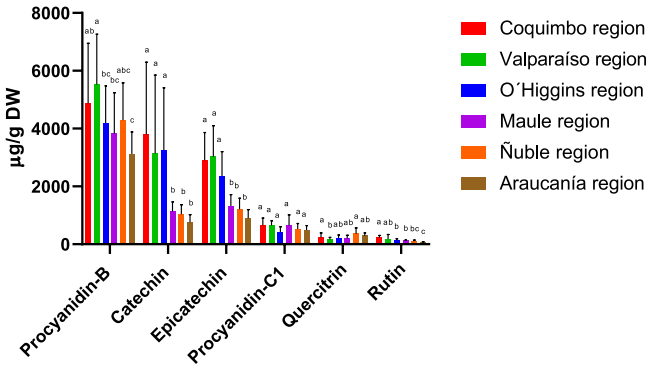

Fig. 2. Phenolic compounds in the leaves of six populations of *P. boldus* (µg/g DW; n = 10). Different letters in columns correspond to significant differences according to Tukey's Test ($p < 0.05$).

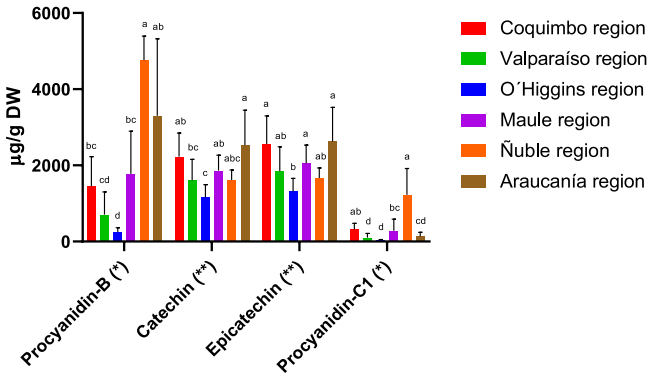

Fig. 3. Phenolic compounds in the bark from six populations of *P. boldus* (µg/g DW; n = 10). Different letters in columns correspond to significant differences according to *Tukey's test ($p < 0.05$), * * Kruskal Wallis test ($p < 0.05$).

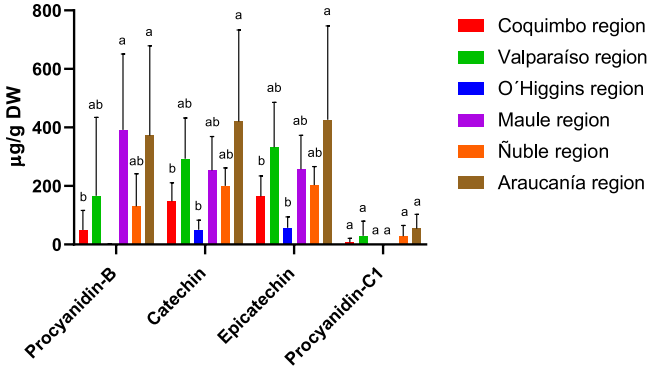

Fig. 4. Phenolic compounds in the wood of six populations of *P. boldus* (µg/g DW; n = 10). Different letters in columns correspond to significant differences according to Tukey's Test ($p < 0.05$).

differences in the total mass of leaves produced (Table S2). The 3.5-year-old saplings grown in the less shaded area had significantly higher contents of phenolic compounds (Fig. 5).

3.2. Alkaloids

Concerning the alkaloidal composition of the leaves, three populations, from the Valparaiso, O'Higgins, and Maule Regions, were particularly rich in coclaurine. The major alkaloid from the Coquimbo Region trees was *N*-methyl-laurotetanine. Laurotetanine contents rose towards the south, while *N*-methyllaurotetanine seemed to follow the opposite trend. Laurolitsine contents also rose southwards. Isocorydine, which was higher towards the north, was second in abundance in the Coquimbo and Valparaíso trees (Fig. 6). We found moderate correlations between the concentrations of some alkaloid pairs: between laurolitsine and laurotetanine ($R^2 = 0.76$), between isocorydine and *N*-methyllaurotetanine ($R^2 = 0.65$) and particularly for isocorydine and *N*-methylcoclaurine ($R^2 = 0.88$) (Table S3).

In the bark, boldine is the most significant alkaloid in many individuals, but not in all since in the branches of the trees sampled in the Valparaíso Region coclaurine was most abundant. At the same time, significant differences were found at the population level for the concentrations of other alkaloids, such as laurolitsine, laurotetanine, and isocorydine. Coclaurine and boldine decreased drastically towards the south (Fig. 7). The correlation between coclaurine and boldine ($R^2 = 0.92$) was particularly striking, but significant correlations were also found between the concentrations of *N*-methylcoclaurine and boldine ($R^2 = 0.82$) and *N*-methylcoclaurine and coclaurine ($R^2 = 0.89$) (Table S4).

In the wood of five populations, laurolitsine was the most abundant alkaloid, followed by coclaurine, laurotetanine, boldine, and reticuline. Besides, more than 1000 µg/g DW of each of these four alkaloids were found in some individuals. Important differences were noted at the population level. For example, in the Valparaíso sample, the mean concentration of boldine was three times the value found in the (more southerly) Maule trees and four times that for the (northern) Coquimbo population. The trees from the Valparaíso Region also showed particularly high values for reticuline, *N*-methylcoclaurine, and isocorydine relative to the other regions. Instead, the individuals growing in the Coquimbo Region showed higher values for laurolitsine than any others except those from the (southernmost) Araucanía Region (Fig. 8). The most interesting correlation to point out for the wood alkaloids was between coclaurine and *N*-methylcoclaurine ($R^2 = 0.82$). The latter also showed moderate correlations with reticuline ($R^2 = 0.55$) and laurolitsine ($R^2 = 0.60$) (Table S5).

The plants growing under 80 % shade contained significantly more boldine, isocorydine, *N*-methyl laurotetanine, reticuline, higenamine, and *N*-methylcoclaurine than those growing under 40 % shade. Decreased shading was only associated with increased laurolitsine, while laurotetanine and coclaurine were unchanged. The coclaurine concentration was similar to those found in the Valparaíso, O'Higgins, and Maule populations. The main alkaloids found in the 80 % and 40 % shade saplings were *N*-methyllaurotetanine and laurotetanine respectively (Fig. 9).

The individuals that had grown under 40 % shade were evaluated the following year. We found that boldine, isocorydine, *N*-methyl laurotetanine and higenamine increased significantly, while laurolitsine,

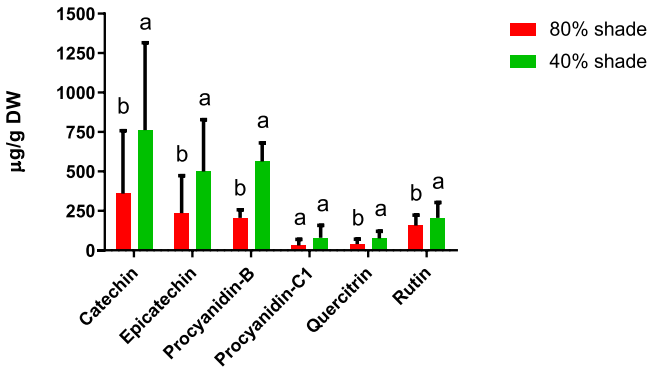

Fig. 5. Phenolic compounds in the leaves of 3.5-year-old individuals of *P. boldus* depending on the shading level (µg/g DW; n = 25). Different letters in columns correspond to significant differences according to Tukey's Test ($p < 0.05$).

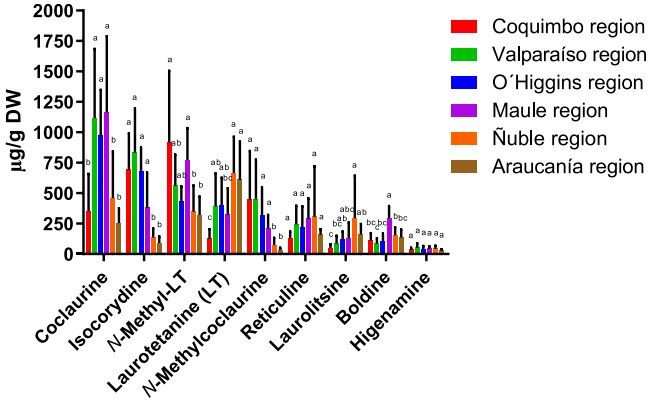

Fig. 6. Alkaloidal contents in the leaves of six populations of *P. boldus* (µg/g DW; n = 10). Different letters in columns correspond to significant differences according to Tukey's Test ($p < 0.05$).

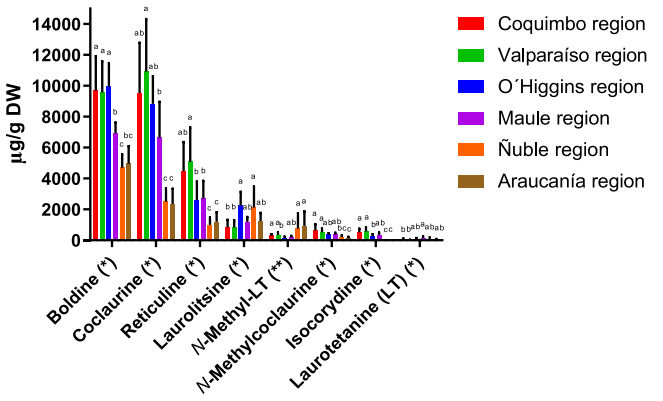

Fig. 7. Alkaloidal contents in the bark of six populations of *P. boldus* (µg/g DW; n = 10). Different letters in columns correspond to significant differences according to Tukey's Test ($p < 0.05$), ** Kruskal Wallis test ($p < 0.05$).

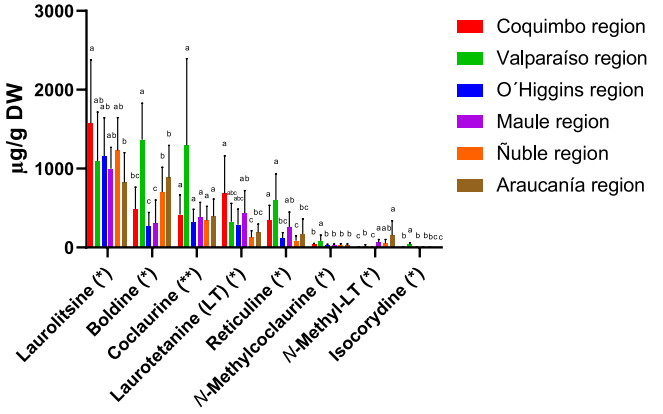

Fig. 8. Alkaloidal contents in the wood of six populations of P. boldus (µg/g DW; n = 10). Different letters in columns correspond to significant differences according to *Tukey's test ($p < 0.05$), ** Kruskal Wallis test ($p < 0.05$).

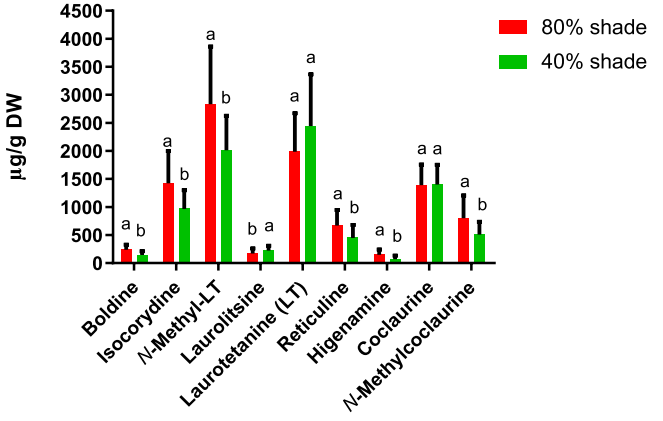

Fig. 9. Alkaloidal contents in the leaves of 3.5-year-old individuals of *P. boldus* depending on the shading levelµg/g DW; n = 25). Different letters in columns correspond to significant differences according to Tukey's test ($p < 0.05$).

laurotetanine and *N*-methyllaurotetanine decreased (Fig. 10). The decrease in laurotetanine and the increase in *N*-methyllaurotetanine is striking, as there seems to be a compensatory, possibly age-related effect. Although not so noticeable, the increase of boldine and decrease of laurolitsine might be a reflection of a similar effect. Notably, in both cases the change implies an increase in *N*-methylation. Coclaurine remained unchanged in both analyses.

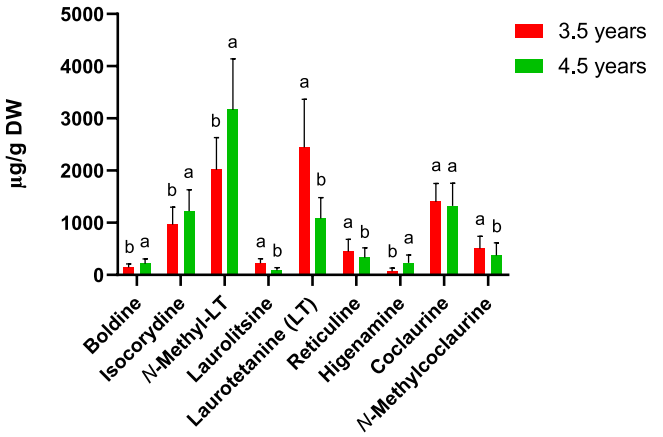

Fig. 10. Alkaloidal contents in the leaves of 3.5 and 4.5-year-old individuals of *P. boldus* farmed under 40 % shade conditions (µg/g DW; n = 25). Different letters in columns correspond to significant differences according to Tukey's test ($p < 0.05$).

3.3. Essential oils

Comparing all six wild populations, there were no important differences in their EO composition. The main EO components were *p*-cymene, ascaridole, and 1,8-cineole. The only noteworthy features were a higher *p*-cymene content in the sample from the Ñuble Region, sabinene in the Maule sample, and α-terpinyl acetate in the Coquimbo sample (Fig. 11). A graph with the individual value of each component is also presented (Fig. S5).

In trees regularly harvested in the wild, the main EO components were ascaridole (16.2 %), *p*-cymene (15.5 %), and carvenone oxide (10.3 %). Similar results were found in the trees that had not been harvested for many years: *p*-cymene (16.9 %), ascaridole (14.9 %), and carvenone oxide (9.2 %). In the farmed saplings, *p*-cymene (18.5 %), α-terpinene (14.3 %) and 1,8-cineole (10.5 %), γ-terpinene, spathulenol, 3-thujene, and linalool predominated, and interestingly, ascaridole, carvenone oxide, cyclooctanone, and 4-terpineol were less abundant in the cultivated trees than in those growing wild (Fig. 12).

4. Discussion

Pharmacopoeias tend to base the identification and quality assessment of boldo leaves, on their alkaloid content, and specifically that of boldine. However, as upon fractionation of the leaf extract the antioxidative activity was found to be mainly due to the flavonoid fraction (44.1 %), followed by the alkaloids (15.6 %) (Quezada et al., 2004), we

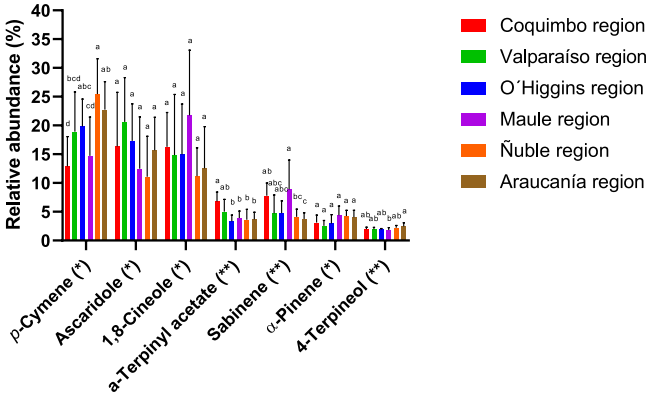

Fig. 11. Main volatile compounds in the leaf EOs of six populations of *P. boldus* (n = 10). Different letters in columns correspond to significant differences according to * Kruskal Wallis test ($p < 0.05$),**Tukey's test ($p < 0.05$).

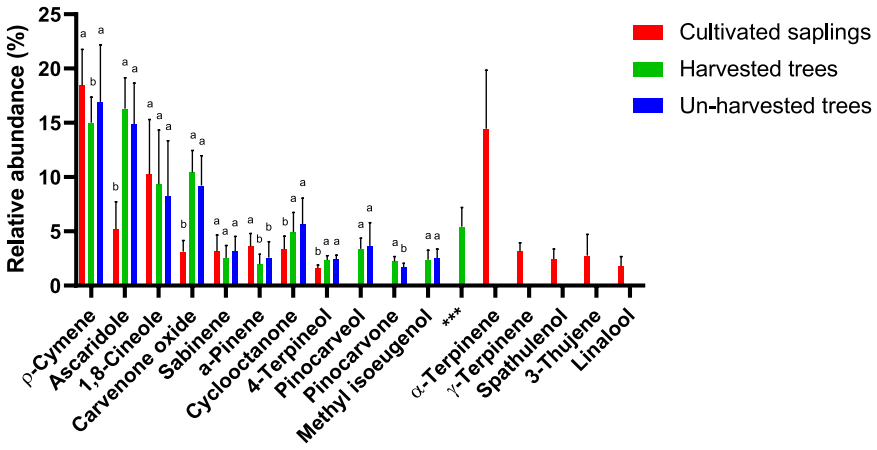

Fig. 12. Main volatile compounds in the leaf EOs of *P. boldus* harvested (n = 30), non-harvested (n = 20), and farmed (40 % shade, n = 25). Different letters in columns correspond to significant differences according to Tukey's Test ($p < 0.05$). (***): 5,9,9-trimethyl-spiro[3,5]non-5-en-1-one.

would recommend a more comprehensive analysis including phenolics.

In line with this, we must keep an open mind while studying the secondary metabolites of *P. boldus*, as not only its boldine content but a panoply of alkaloids, polyphenols, and EO components make significant contributions to its biological activities (Cassels et al., 2021, 2019). Identified compounds such as catechin, epicatechin, and procyanidins should be quantified (Falé et al., 2012; Quezada et al., 2004; Schmeda-Hirschmann et al., 2003; Simirgiotis and Schmeda-Hirschmann, 2010; Zielinski et al., 2014). Even the volatile fraction of the infusions (de Souza et al., 2019) should be analyzed, since aspects such as the concentration in the leaves and the type of extraction (aqueous/hydroalcoholic) determine what is ingested by the consumer or how these secondary metabolites are to be used industrially.

In the present work, we found a similar, more detailed pattern to that described previously by us (Fuentes-Barros et al., 2018). Boldine is not the main leaf alkaloid, and in abundance it lies below coclaurine, *N*-methyllaurotetanine, isocorydine, or laurotetanine, the contents of all of which vary considerably. Instead, boldine is almost always the main alkaloidal component of the bark, which is used as its industrial source. Publications addressing the natural variability of alkaloid-bearing plants are increasingly common. For example, great differences were found in the pyrrolizidine alkaloids from different organs of *Senecio vulgaris* L. (Asteraceae) plants (Cheng et al., 2017), quinolizidine alkaloids from different organs and different species of *Sophora* spp. grown at a single site (McDougal et al., 2015), and for the bark alkaloids of different stands of *Cinchona calisaya* Wedd. (Rubiaceae) (Maldonado et al., 2017). Regarding aporphine alkaloids, this has been recorded previously in *P. boldus* (Fuentes-Barros et al., 2018), in *Cryptocarya alba* (Molina) Looser (Lauraceae) (Castro-Saavedra et al., 2016; Giordano et al., 2019), in the leaves, stems, and rhizomes of *Cissampelos capensis* L.f. (Menispermaceae) (de Wet et al., 2011) and in different batches of *Litsea cubeba* (Lour.) Pers. (Lauraceae) roots (Zhang et al., 2015). The same has been seen for non-aporphine benzylisoquinoline alkaloids in *Argemone mexicana* L. (Papaveraceae), *Corydalis cheilanthifolia* Hemsl. (Papaveraceae), *Berberis thunbergii* DC. (Berberidaceae), *Nigella sativa* L. (Ranunculaceae), *Papaver bracteatum* Lindl. (Papaveraceae) and *Sanguinaria canadensis* L. (Papaveraceae) (Hagel et al., 2015). In these last six species and *C. alba*, alkaloid fluctuations appear to be greater than for non-alkaloidal phenolics (Giordano et al., 2019), like some of the results of the present work.

An evolving market for medicinal herbs may be expected to more highly value products richer in particular constituents. Therefore, disregarding their non-alkaloidal phenolic and essential oil contents, boldo products with higher concentrations of alkaloids and specifically boldine may be more desirable from a commercial standpoint. This would seem to favor the exploitation of trees growing wild in the more northerly locations which are precisely those most threatened by changes in land use, climate change and wildfires (Smith-Ramírez et al., 2023). It is worth mentioning that the alkaloid laurolitsine is relatively abundant in wood from all the populations studied by us. Boldo wood is a neglected resource which is often burnt for charcoal. In recent years this alkaloid has been found to ameliorate conditions such as osteoporosis and rheumatoid arthritis (Cassels et al., 2019), suggesting that wild-harvested boldo could be exploited more rationally. According to our results, boldo leaves from farmed saplings have a higher concentration of bioactive alkaloids than those collected from wild populations. These data also strongly suggest cultivation as a very attractive alternative for industrial production while diminishing pressure on threatened ecosystems. The antioxidant flavonoid catechin, which may be considered a biomarker of the leaves of this species (Quezada et al., 2004), has been found in highly varied concentrations (Pacheco, 2011; Schmeda-Hirschmann et al., 2003). Extracts rich in other phenolics such as kaempferol, quercitrin, and quercetin (Klimaczewski et al., 2014), 3, 4-dihydroxybenzoic acid (Bianchini et al., 2016), and procyanidins B3 and C2 (Pastene et al., 2014), and B1 (Zielinski et al., 2014) have also been identified. Our present results confirm wide ranges for catechin, the mixture of procyanidins B1 and B2, epicatechin, and procyanidin C1. Concerning non-alkaloidal phenolic compounds, the leaves collected from the more southerly populations were poorer in these metabolites. Also, the leaves of the farmed individuals in this work contained less non-alkaloidal phenolics than the leaves of the wild trees, particularly when grown under darker shade. The decrease now seen for *P. boldus* in the concentration of catechin and epicatechin due to shading has also been found in tea, *Camellia sinensis* (L.) Kuntze (Theaceae) (Liu et al., 2018; Wang et al., 2012; Zhang et al., 2014).In leaves, 80 % vs. 40 % shade seems to be more favorable and there is a significant increase in the content of some alkaloids with age., future commercial ventures farming boldo should consider the tradeoff between potentially valuable flavonoids and the more sought-after alkaloids.

The yields and relative concentrations of the leaf essential oil constituents are extremely variable (Cassels et al., 2019), between 2 % and 0.01 % (de Castro et al., 2016; de Souza et al., 2019; Mazutti et al., 2008; Sargenti and Lanças, 1997; Urzúa et al., 2010). Unpublished results from our laboratory lie in an intermediate range (0.61 % ± 0.42). The oil usually contains a high proportion of *p*-cymene, and the toxic ascaridole is thought to be characteristic (European Medicines Agency, 2016; European Pharmacopoeia, 2013; Fundación Farmacopea Chilena and Universidad de Valparaíso, 2016). Ascaridole may be present in large concentrations, e.g. 24 % (Silva-Aguayo et al., 2021), 31 % (Blázquez and Carbó, 2015) or even 51.2 % (Verdeguer et al., 2011), making boldo leaves a very important source of this compound (de Castro et al., 2016). Nevertheless, individuals with low ascaridole content have also been

found, down to 7 % or less (Rezende et al., 2017; Urzúa et al., 2010; Vila et al., 1999) or even traces (de Souza et al., 2019; Viana et al., 2020), including the present work. Some samples are rich in α-terpinene (Passone and Etcheverry, 2014), specifically the farmed saplings analyzed here, or limonene dioxide (Mazutti et al., 2008). Here again, shading seems to diminish the content of the potentially undesirable ascaridole, adding to the attractiveness of boldo farming.

However, the age of the trees and how they are farmed, specifically with an optimal light/shade ratio, may play an important role and might increase the alkaloid and polyphenol yield. Many other cultural factors must be evaluated, starting with the use of fertilizers and water restriction among other variables. Boldo bark is a highly valuable source of boldine, reticuline, and coclaurine, and this work opens up a new prospect considering the previously unknown procyanidin content of south-central Chilean populations.

5. Conclusion

In this study we have confirmed the value of the aerial biomass of *Peumus boldus* as an important source of secondary metabolites, no longer limited to the well-known alkaloids but also considering its phenolic compounds and essential oil constituents. The leaves contain high concentrations of both alkaloidal and non-alkaloidal phenolic compounds with high antioxidant potential. The boldine-rich bark also contains valuable amounts of procyanidins. The wood, which is usually discarded, is an exceptional source of laurolitsine, by far the main alkaloid in this abundant and neglected resource. The considerable variability within each population and among populations for a significant number of compounds classically documented for the species, in leaves, wood, and bark, is probably due to the interplay of multiple factors, clearly beyond the differences in climate and soils found throughout the range of this tree, as the genetic variability of the species may contribute, and the development of local ecotypes cannot be excluded. The north-central wild populations showed higher concentrations of alkaloids and polyphenols in leaves and alkaloids in the bark compared to more southern populations. Saplings farmed under different shade conditions contained higherless leaf polyphenolic concentrations with increasing lightshade while most some alkaloids increased with the shade. When analyzed the following year, higenamine, boldine, isocorydine and *N*-methyllaurotetanine increased. The principal components of the leaf essential oils from the wild populations were p-cymene, ascaridole and 1,8-cineole, while in the farmed trees ascaridole was replaced by its precursor α-terpinene as the second most abundant constituent. Therefore, as boldo is widely used as a medicinal herb, as a flavorizer, and as a source of boldine, routine quality control and standardization of products put on the market should increase their value considerably. Furthermore, the leaves, wood, and bark obtained from farmed individuals should be seen as alternative commercial products and for the production of alkaloids, reducing the present threatening pressure on trees growing in the wild. The relatively high alkaloid content of farmed leaves and the absence of ascaridole make them particularly attractive for an increasingly demanding and discerning market.

Declaration of Competing Interest

Gonzalo Fuentes-Barros, Antonia González-Cooper and Sebastián Castro Saavedra are partners in SAPHYCHEM (South American Phytochemical), a company that markets boldo products.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jarmap.2023.100502.

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