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Variation of the alkaloid content of *Peumus boldus* (boldo)

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ABSTRACT

Eighteen alkaloids were detected in the bark, leaves, wood and roots of *Peumus boldus*, including traces of secoboldine, *N*-methylsecoboldine (boldine methine), glaucine and norreticuline, not reported previously as constituents of this species. Using appropriate standards, we quantified thirteen of them by UHPLC-MS/MS. Boldine was dominant in the bark, and laurolitsine in wood and roots. The alkaloid composition of the leaves, determined for 130 individually identified trees, classified by age and sex, was highly variable, where *N*-methyllaurotetanine, laurotetanine, coclaurine and in some cases isocorydine predominated, but not boldine.

1. Introduction

Peumus boldus Mol. (*Monimiaceae*; 'boldo' in the vernacular and internationally) is an endemic tree of central Chile, where it dominates the landscape of many parts of the Mediterranean climate zone [1,2]. Boldo has developed physiological mechanisms that allow it to tolerate drought, high temperatures and strong solar irradiation [3,4] and the ability to regrow from the roots after felling or burning, making it appropriate to colonize land that has suffered desertification processes [5]. Archeological findings show that the aromatic boldo leaves were chewed for unknown purposes by some of the earliest inhabitants of South America 14,600 years ago [6], and its bark was smoked, apparently in a ritual context, 1000–1500 years ago [7]. Traditional medicinal applications include earache, headache, rheumatism, nasal congestion and, prominently, digestive and biliary disorders [8]. Boldo leaves are arguably one of the most valuable non-timber forest products in central Chile, currently attracting an export income of around five million US dollars per year [9]. Their widespread use outside Chile, mainly for dyspepsia and mild digestive spasms, for its hepatoprotective, choleretic and cholagogic properties, and also as a mild sedative, has led to their inclusion in the European Pharmacopoeia (*boldi folium*) [10] and their recent assessment by the European Medicines Agency (EMA) [11]. While clinical studies are lacking, the traditional use of boldo leaf infusions as an aid to digestion seems justified [12]. While many studies attribute the beneficial effects of boldo to its content of boldine [8,12,13], considered its most characteristic alkaloid, the remarkable content of polyphenols in this species [14], especially catechin and related compounds [15,16], plus relevant concentrations of other phenolic alkaloids [8], suggest a more complex interpretation. In this regard it is interesting to note that the documented antiinflammatory activity of boldine [17], is potentiated when this alkaloid is co-administered with reticuline, also present in boldo [18].

The alkaloid footprint of boldo leaves has been studied for decades, initially adding the aporphines isocorydine, *N*-methyllaurotetanine and norisocorydine [19] to boldine, which had been found almost a century before [20]. Some years later the 1-benzyl-1,2,3,4-tetrahydroisoquinoline reticuline, and the aporphines isoboldine, laurotetanine, laurolitsine and isocorydine *N*-oxide were identified [21–23]. Early use of HPLC led to the quantification of some of these (only tentatively identified from their retention times) in boldo leaves and extracts purchased from European suppliers, where boldine was usually found to be a relatively minor alkaloid [24,25]. This basically unchanged methodology is dictated by the European Pharmacopoeia, where boldine is still considered a reference [10]. It is also surprising that the EMA should state that "boldine is usually the major alkaloid" [11]. Quite recently the benzylisoquinoline *N*-methylcoclaurine and the noraporphine norglaucine [26], and in a particularly thorough investigation, the benzylisoquinolines *N*-methylcoclaurine, reticuline, the aporphine norisocorydine, isocorydine, *N*-methyllaurotetanine, boldine and laurotetanine, the proaporphines glaziovine and pronuciferine, and

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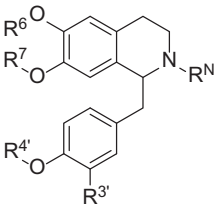
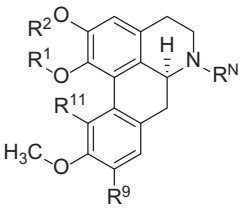
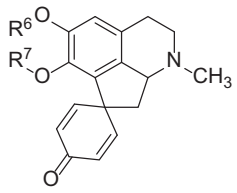
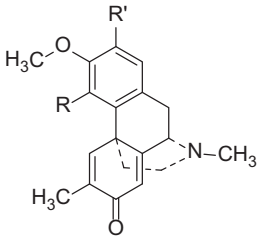


R^6	R^7	R^N	$R^{3'}$	$R^{4'}$	
H	H	H	H	H	Higenamine
CH_3	H	H	H	H	Coclaurine
CH_3	H	CH_3	H	H	*N*-Methylcoclaurine
CH_3	H	CH_3	OH	CH_3	Reticuline
CH_3	H	H	H	CH_3	Norreticuline

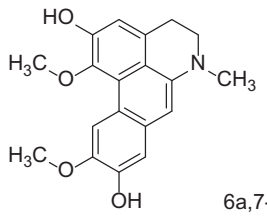


R^1	R^2	R^N	R^9	R^{11}	
H	CH_3	CH_3	OH	H	Isoboldine
CH_3	H	H	OH	H	Laurolitsine
CH_3	H	CH_3	OH	H	Boldine
CH_3	CH_3	H	OH	H	Laurotetanine
CH_3	CH_3	CH_3	OH	H	*N*-Methyllaurotetanine
CH_3	CH_3	H	OCH_3	H	Norglaucine
CH_3	CH_3	CH_3	OCH_3	H	Glaucine
CH_3	CH_3	CH_3	H	OH	Isocorydine
CH_3	CH_3	H	OCH_3	OH	Norisocorydine

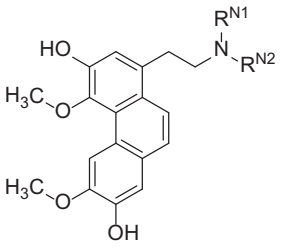




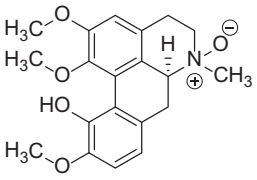
R^6	R^7		R	R'	
CH_3	H	Glaziovine	OH	H	Sinoacutine
CH_3	CH_3	Pronuciferine	H	OH	Pallidine



6a,7-dehydroboldine



R^{N1}	R^{N2}	
H	CH_3	Secoboldine
CH_3	CH_3	*N*-methylsecoboldine



R^1	R^2	R^N	R^9	R^{11}	
CH_3	CH_3	CH_3	H	OH	Isocorydine N-oxide

Fig. 1. Alkaloids identified from *Peumus boldus*.

the morphinandienones pallidine and sinoacutine were isolated from the leaves [27]. See Fig. 1.

Boldo bark has been known for decades to be a good commercial source of boldine [8], which is clearly the dominant alkaloid in this tissue. A more detailed study revealed the presence of *N*-methyllaurotetanine, isocorydine and norisocorydine plus pronuciferine, sinoacutine, and 6a,7-dehydroboldine [28,29]. Both coclaurine enantiomers were subsequently identified in the bark [30]. Boldo wood was examined for alkaloids in two undergraduate theses, which concluded that the noraporphine laurolitsine (norboldine) was the major component together with smaller amounts of boldine, laurotetanine and several unidentified bases [31,32].

The HPLC analyses of several samples of boldo leaves and extracts found laurotetanine or *N*-methyllaurotetanine as the major alkaloids, often followed by isocorydine [25,26]. However, an analysis of leaves collected in central-southern Chile and of boldo tea bags indicated isocorydine, with less *N*-methyllaurotetanine, as the major alkaloids [33], while a ^{13}C NMR profile of a crude boldo leaf extract suggested that norisocorydine was the most abundant, also followed by *N*-methyllaurotetanine [34]. Taken together, these literature results suggest that the alkaloid profile of *Peumus boldus* is highly variable. As the medicinal properties of boldo leaves are presumably affected by such differences, we decided to address the variability of the alkaloidal composition of the leaves, extending our quantitative analysis to the alkaloids present in the bark, wood and roots of boldo trees.

2. Materials and methods

2.1. Plant material and chemicals

Leaves, bark, wood and roots of *P. boldus* were collected in Spring-Summer 2014–2015 near María Pinto (33°26′ S and 71°18′ W, 230–250 m above sea level), Santiago Metropolitan Region, Chile. Voucher specimens 181,128, identified by Alicia Marticorena, are deposited in the Herbarium of the Botany Department, University of Concepción, Concepción, Chile. Leaves were collected from trees growing in an area (MP-1) where the leaves are harvested regularly every five years, from a contiguous area where no harvesting has taken place in recent decades (MP-2) and, for control purposes, from another harvested area (MP-3) nearby, all with trunk diameter at breast height (DBH) < 40 cm and usually about 15 cm. The leaves and bark of older trees (DBH > 40 cm) were collected in a relatively undisturbed ravine (LA) a few kilometers further north and higher up (33°23′ S and 70°59′ W, about 570 m above sea level). The four-year old saplings were from an experimental farm in San Bernardo, Santiago Metropolitan Region (33°39′ S and 70°43′ W, about 550 m above sea level). Silica gel 60 Å (40–63 μm) was purchased from Sigma-Aldrich. Anhydrous sodium sulfate was purchased from Merck. All solvents used were of analytical grade or double distilled. Water was of ultrapure grade.

2.2. Extraction for alkaloid isolation

Dried and ground bark, wood, roots and leaves from a single tree growing next to MP-2 (5, 10.0, 4.0 and 2.0 kg respectively) were submerged three times for 4 h each time in MeOH (5 L/kg) at 50 °C. The extracts from each organ were combined, filtered and concentrated to yield dark gummy materials. These were suspended in DCM and extracted with 1 M aqueous HCl. The aqueous phase was washed with hexane, EtOAc and DCM, then made basic (pH 9–10) with concentrated aqueous ammonia solution (25%) and finally extracted three times with DCM. The combined extracts were dried with Na_2SO_4, filtered and concentrated yielding 35.5, 4.0, 3.5, and 0.5 g of total alkaloids for bark, wood, roots and leaves respectively. Aliquots of these extracts were kept for UHPLC-MS/MS analysis as described below.

2.3. Isolation and identification

Each total alkaloid extract was fractionated chromatographically (30 × 3, 30 × 5 and 30 × 7 cm diameter columns) using silica gel 60 Å (40–63 μm). The elution was carried out with an EtOAc:MeOH gradient from pure EtOAc to pure MeOH, collecting 100 ca. 20 mL fractions. All fractions were monitored by TLC (EtOAc:MeOH 4:1 and/or DCM/MeOH 5:3) examining under UV light (254 and 365 nm) and applying Dragendorff's reagent to detect the presence of alkaloids. The isolated alkaloids were identified by ^{1}H NMR and mass spectrometry and by chromatographic comparison with standards.

2.4. Reference standards

Purity of all standards was over 98% by HPLC. Glaucine was prepared from boldine by methylation with diazomethane [35,36]. Boldine was isolated from the bark of *P. boldus*, laurolitsine from the wood and laurotetanine from the leaves. Boldine was converted into secoboldine and *N*-methylsecoboldine (boldine methine) as described in the literature [37,38]. Racemic norreticuline, coclaurine and *N*-methylcoclaurine were synthesized by the Bischler-Napieralski route [39]. Racemic norcoclaurine (higenamine) was prepared by demethylation of coclaurine [40]. Isocorydine HCl was purchased from PhytoLab GmbH & Co. Reticuline was isolated as described previously from the bark of *Cryptocarya alba* [41], and additional laurotetanine was isolated from the bark of *Laurelia sempervirens* [42].

2.5. Sample preparation for UHPLC

Boldo leaves (50 g fresh weight) were harvested from each tree, oven-dried at 40 °C for 72 h, ground and passed through a 2 mm screen. The screened material (1 g) was extracted (5 times) with MeOH (100 mL each time) for 2 h at 60 °C, filtering each time through Whatman N°1 paper, and the extract was concentrated to dryness in a rotary evaporator. The residues were resuspended in MeOH (20 mL) and stored in amber vials until use.

2.6. Liquid chromatography and mass spectrometry

All the samples were analyzed by UHPLC-MS/MS in an EkspertUltraLC 100-XL ultra-high pressure liquid chromatograph coupled to an electrospray (ESI) ABSciex Triple Quad 4500 triple quadrupole mass spectrometer. A PhenomenexSynergi™ Fusion-RP 80 Å (50 mm × 2.0 mm, 4 μm) column was employed and the mobile phase was prepared from aqueous formic acid 0.1% v/v (eluent A) - acetonitrile (eluent B). The reported gradient program [43] was used with some modifications: (time, min/%B) 3/3%, 13/15%, 16/20%, 17/3%, 20/3%, and the injection volume was 10 μL. The mass spectrometer parameters were: gas 1 N_2 (40 psi); gas 2 N_2 (50 psi); ion spray voltage, 3500 V, ion source temperature, 650 °C; curtain gas N_2 (25 psi), flow 0.3 mL/min and scan mode MRM with positive polarity. The UHPLC-MS/MS system was controlled with Analyst 1.6.2 and the data processed with Multiquant 3.0. Calibration curves were built for each compound in the 0.1–0.8 μg/mL range.

2.7. Validation of the UHPLC–MS/MS method

Stock solutions of the reference standards were prepared in metanol (1 mg/mL). These were diluted to 8 different concentrations from 0.1 to 0.8 μg/mL. The calibration curve was built showing the concentrations of each analyte vs. the corresponding peak areas. The limit of detection (LOD) and the limit of quantification, intraday and interday precision, stability, repeteability and recovery were determined according to the "Guidance for industry, Q2B Validation of Analytical Procedures: Methodology" [44]. Intraday precision was determined with three repeats on the same day, and interday precision with three repeats on three successive days, both expressed as percent relative standard deviation (RSD). Stability was assessed from six independent analyses at 0, 7, 10, 14, 36 and 44 h, expressed as RSD. Repeteability se determined from six repeats and was also expressed as RSD. The recovery test was done adding three concentrations of standard (low, medium and high) to a boldo leaf sample, and the results are expressed as the mean RSD and according to the formula for the mean recovery percentage:

$$(\%) = 100\,(\text{observed amount} - \text{original amount})/\text{added amount}$$

2.8. Statistical analysis

Statistical calculations were carried out with Minitab® version 16.0 (Minitab Inc., State College, PA, USA) and Infostat® version 2017 [45] for Windows. The descriptive statistics were determined for each parameter of interest and the residuals were subjected to Anderson-Darling normality test and Bartlett's homoscedasticity test to decide whether parametric or non-parametric analyses were appropriate. When a distribution was significantly normal, a general linear model was used to evaluate the different factors and their interactions, and Tukey's multiple comparison method was used for inter-group comparisons. When normality was absent, Box-Cox and Johnson transformations were performed, and if no transformation proved adequate for normalization, a Mann-Whitney or Kruskal Wallis test was used to compare independent samples.

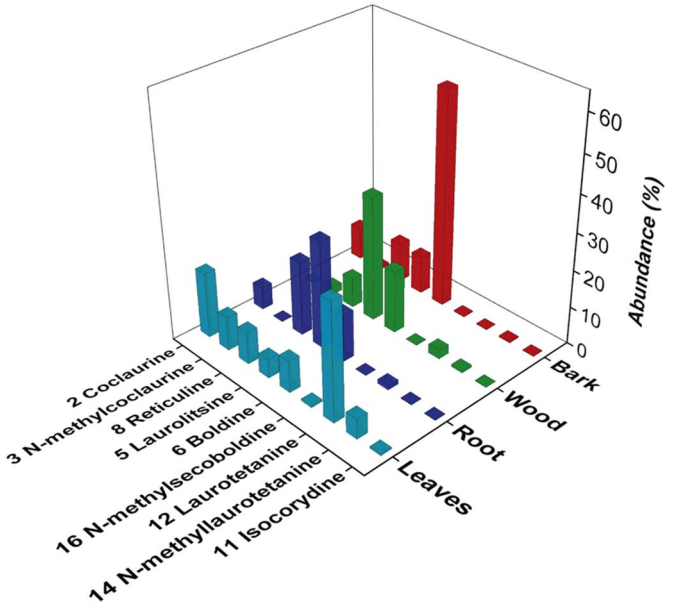


Fig. 2. Alkaloidal composition of crude basic extracts isolated from different parts of a single *P. boldus* tree. The numbers correspond to the order in which the alkaloids elute from a C_{18} chromatographic column.

3. Results and discussion

We did a preliminary, classical analysis of leaves, wood, bark and roots of a single tree (see Experimental). These plant parts afforded (based on dry weight) 0.025, 0.04, 1.0 and 0.09% of crude alkaloids, respectively. These fractions were extracted following the guidelines of the European Pharmacopoeia, and the extracts were analyzed by UHPLC-MS/MS (Fig. 2 and 3, Table 1S) and were used for isolation of the major bases.

The most abundant alkaloid detected in the bark was boldine (around 60% of the total alkaloids), as reported previously [8], although the yield of total alkaloids in our material was much lower than some reported values that go up to 7.4–7.7% [32,46–48]. It seems possible that the higher yields reported earlier were obtained from the scaly bark of old trees which are now very rare, while the material we analyzed was from smooth branches of younger individuals. The high but rather variable concentration of boldine in this tissue and the

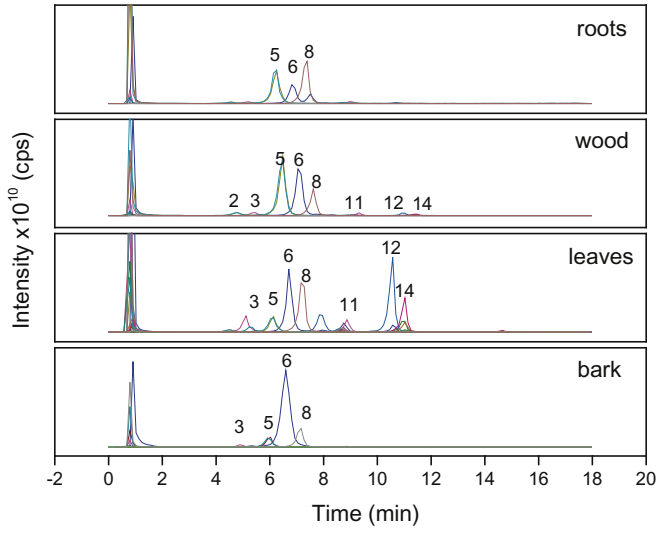


Fig. 3. Typical UHPLC chromatograms obtained from the different organs of *P. boldus* trees. Numbering corresponds to alkaloids in Table 2S.

Table 1
Mean leaf alkaloid contents of *P. boldus* from MP-1in successive years (μg/g DW ± SDM).

N	Compound	Late Spring 2014 n = 30	Late Spring 2015 n = 30
5	Laurolitsine	136.4 ± 99.1[a]	152.9 ± 99.1[a]
6	Boldine	120.5 ± 71.8[b]	177.6 ± 72.2[a]
8	Reticuline	184.4 ± 112.0[b]	466.3 ± 348.4[a]
11	Isocorydine	325.3 ± 211.4[b]	447.1 ± 251.7[a]
12	Laurotetanine	550.3 ± 236.3[a]	515.6 ± 265.5[a]
14	*N*-methyllaurotetanine	382.3 ± 224.9[b]	960.9 ± 374.9[a]
16	*N*-methylsecoboldine	40.5 ± 30.8	Nd

Means for each individual alkaloid with different superscripts (a or b) in both columns are significantly different. Tukey test $p < .01$; nd: not determined.

generally lower concentrations of other alkaloids (tetrahydroisoquinolines and aporphines) are noteworthy and quite different from their distribution in other parts of the tree. From these results it may be conjectured that boldine plays a functional role in the bark, probably protecting it against fungal or insect attack. Laurolitsine and boldine are present in similar amounts in the trunk and root wood, where they are more abundant than other alkaloids.

It may be mentioned that we isolated traces of 6a,7-dehydroboldine from the bark. Although this compound had been recorded as a boldo bark alkaloid over 30 years ago [29], in our opinion, it is most likely formed by air oxidation of boldine and is not an actual metabolite of *P. boldus*. To evaluate this possibility air was bubbled through a solution of pure boldine in methanol for five days at room temperature, after which 6a,7-dehydroboldine could be isolated in 15% yield. This is probably also the case of other oxidized derivatives of the major aporphines of different plants. Two recent examples are 6a,7-dehydronuciferine and 6a,7-dehydroglaucine, found respectively in *Nelumbo nucifera* [49] and *Glaucium flavum* [50].

We also found small amounts of *N*-methylsecoboldine in the bark, and the UHPLC-MS/MS analyses of the different tree parts revealed peaks which we identified as this compound and, in some cases, as secoboldine. Both substances may be formed by Hofmann elimination of (protonated) boldine and of the yet undetected *N*-methylboldinium (we are not aware of any search for quaternary alkaloids in boldo), and we therefore think that they might be artifacts of isolation, as suggested for other 1-aminoethylphenanthrene alkaloids 30 years ago by Shamma and Rahimizadeh [51]. Our analysis showed the presence of one more compound with molecular weight 314 g/mol (possibly norisoboldine) and another three unidentified substances with *m/z* 341. Laurotetanine and coclaurine, at 32 and 18% respectively of the crude bases, were the most abundant alkaloids in the leaves of the sample used for isolation.

We used UHPLC-MS/MS to analyze the alkaloids of leaves, bark, trunk and root wood of *Peumus boldus* trees growing wild or cultivated in the Santiago Metropolitan Region of Central Chile. As boldo leaves are the primary commercial product of this species and they are widely used for their medicinal properties, we dwelt particularly on their interindividual variation in leaves from 130 different trees growing in the Metropolitan Region. We considered the age and sex of the trees (*P. boldus* is a dioecious species) whether the leaves were old or new, whether they grew on old or new shoots (which are cut every few years), and comparing results for two successive years around harvest time (late spring).

UHPLC-MS/MS was used to quantify the concentrations of thirteen alkaloids, in different parts of trees from two neighboring localities (MP-1 and MP-3) in small areas where the leaves are harvested every five years, and another one contiguous to MP-1 (MP-2), where many years have gone by without harvesting. Considering the rather variable total rainfall and distribution previous to and during the spring growing season, it seemed likely that this could be reflected in differences in alkaloid content in the leaves harvested in different years. Consequently, after carrying out the analyses on material from 2014,

Table 2
Mean leaf alkaloid contents in *P. boldus* leaves from MP-1 and MP-2 separated by sex of the tree and age (see text) of the leaves (μg/g DW ± SDM).

N	Compound	Old leaves		New leaves	
		Female n = 25	Male n = 25	Female n = 25	Male n = 25
5	Laurolitsine*	192.5 ± 146.2[a]	125.4 ± 108.0[a,b]	70.9 ± 77.9[c]	110.9 ± 137.5[b,c]
6	Boldine*	151.3 ± 90.7[c]	130.1 ± 101.7[c]	51.3 ± 48.2[a]	82.3 ± 74.9[b]
8	Reticuline**	195.5 ± 108.4[b]	168.3 ± 152.6[b]	100.6 ± 299.8[a]	81.3 ± 97.6[a]
11	Isocorydine*	396.1 ± 262.9[b]	377.7 ± 232.5[b]	464.8 ± 299.6[a]	605.5 ± 363.9[a]
12	Laurotetanine*	531.0 ± 269.6[a]	454.1 ± 245.6[a]	144.3 ± 157.9[c]	192.8 ± 127.4[b]
14	*N*-methyllaurotetanine*	442.9 ± 233.0[a]	364.5 ± 248.2[a]	134.1 ± 234.8[c]	176.4 ± 177.6[b]
16	*N*-methylsecoboldine**	47.2 ± 32.9[b]	39.3 ± 33.2[b]	16.9 ± 32.9[a]	21.8 ± 23.5[a]

Means with different superscripts (a, b or c) in different columns are significantly different *Tukey test ($p < .05$) **Kruskal Wallis test ($p < .05$).

the alkaloid contents of leaves collected from the same 30 individually identified trees from MP-1 in 2015 were compared (Table 1). *N*-Methyllaurotetanine (**14**), reticuline (**8**) and other alkaloids (but not all) were significantly increased in 2015 with regard to 2014.

The issues of the sex of the trees and age of the leaves were addressed analyzing "new" (light green, soft) and "old" (dark green, leathery) leaves of twentyfive male and twentyfive female trees growing in MP-1 and MP-2 (Table 2). Sex differences were apparent in the new leaves of male trees, which were significantly richer in boldine (**6**), laurotetanine (**12**) and *N*-methyllaurotetanine (**14**) than those of female trees. Older leaves contained higher concentrations of most of the alkaloids, with the exception of isocorydine (**11**), but no difference was found between the sexes.

Comparison of the leaf alkaloids from the regularly harvested MP-1, and from MP-2, which have not been harvested for more than a decade, (averages of thirty and twenty trees respectively) indicated that regular harvesting seems to increase higenamine (norcoclaurine) (**1**), reticuline (**8**) and laurotetanine (**12**), and decrease isocorydine (**11**), while boldine (**6**) and other alkaloids are practically unchanged (Table 3).

Nearly every accessible boldo tree has been felled at some time and has thrown up suckers giving it the appearance of a tall shrub. The accepted form of boldo management for leaf (or occasionally bark) harvesting involves cutting down some of the suckers and leaving others while waiting some years for new ones to sprout. This raises the question of whether there is any difference between the leaves collected from old or more recent suckers of the same tree. Our results show that indeed there is a difference, and the leaves from newer suckers are significantly richer in most of the alkaloids (Table 4).

To study the variability of the alkaloid fingerprint in detail, as leaves are the main commercial product of this species, samples from a total of one hundred thirty individually identified trees were analyzed. Our results are summarized in Table 5.

Analyses by Betts [25] and by De Orsi et al. [52] showed low concentrations of boldine (< 0.01%) in boldo leaves, and several other publications gave similar results [28,29,53,54]. Nevertheless, higher values have been reported: 0.016 to 0.059% [55] and 0.01–0.05% [56]. Our average values for mature leaves are close to this range 0.01% to 0.018%, but variations from one tree to another are considerable. In this sense, in the MP-1 + MP-2 population 34% of the trees contained < 0.009% of boldine in the mature leaves and, considering young leaves, 74% of the samples gave an average of 0.0066%. We also found low values in the farmed saplings, 10% of which contained < 0.009% boldine in the leaves. In contrast, the highest values found were for an MP-1 + MP-2 female tree with 0.044% and a single sapling with 0.046%.

It must be stressed again that boldine was not the major leaf alkaloid in our study. *N*-methyllaurotetanine and laurotetanine usually predominated, with coclaurine and isocorydine also standing out. Even *N*-methylcoclaurine and reticuline were often more abundant than boldine, and the concentrations of laurolitsine (norboldine) were similar to those of the erroneously termed "most characteristic" alkaloid of boldo leaves.

A single tree from the MP-2 area was anomalously low in all alkaloids, to the extent that some could not be detected. The highest total alkaloid concentrations were detected in the leaves of farmed saplings, and the same was true for each individual alkaloid. These results suggest that cultivation might be a good choice to generate a sustainable income and reduce the pressure on wild populations that are currently the only commercial source of boldo leaves [9].

To compare the abovementioned (MP) population with much older trees that have not been disturbed for well over twenty years (LA), stem bark and wood were analyzed for alkaloids. In the stem tissues of the trees from the currently exploited population the bark presents higher alkaloid concentrations, where boldine (**6**) and coclaurine (**2**) are the main components, while the wood contains mainly laurolitsine (**5**), coclaurine (**2**), boldine (**6**) and laurotetanine (**12**) in that order. On the other hand, in the old individuals the difference between bark and wood is much less marked, usually due to a lower alkaloid content in

Table 3
Leaf alkaloid concentrations of individual trees harvested < 5 years ago (MP-1) and unharvested (MP-2) trees (μg/g DW ± SDM).

N	Compound	MP-1 n = 30	MP-2 n = 20
1	Higenamine	243.6 ± 184.3[a]	50.5 ± 31.4[b]
2	Coclaurine	993.5 ± 714.1[a]	912.9 ± 508.4[a]
3	*N*-methylcoclaurine	278.7 ± 217.8[a]	369.2 ± 298.9[a]
5	Laurolitsine	152.9 ± 99.1[a]	138.8 ± 119.8[b]
6	Boldine	177.7 ± 72.2[a]	172.2 ± 83.3[a]
8	Reticuline	466.3 ± 348.4[a]	272.2 ± 208.7[b]
11	Isocorydine	447.1 ± 251.7[b]	645.9 ± 293.3[a]
12	Laurotetanine	515.6 ± 265.5[a]	307.9 ± 160.8[b]
14	*N*-methyllaurotetanine	960.9 ± 374.9[a]	936.9 ± 439.2[a]

Mean values with different superscripts (a or b) in boh columns are significantly different. Tukey test ($p < .05$).

Table 4
Leaf alkaloid concentrations of individual trees comparing leaves from suckers ten or more years old and less than five years old (μg/g DW ± SDM).

N	Compound	Old suckers n = 30	New suckers n = 30
1	Higenamine*	243.6 ± 184.3[a]	128.1 ± 110.9[b]
2	Coclaurine*	993.5 ± 714.1[b]	1787.0 ± 632.9[a]
3	*N*-methylcoclaurine*	278.7 ± 217.8[b]	406.4 ± 271.5[a]
5	Laurolitsine*	152.9 ± 99.1[a]	168.8 ± 121.1[a]
6	Boldine*	177.6 ± 72.2[a]	216.6 ± 105.5[a]
8	Reticuline*	466.3 ± 348.4[b]	516.2 ± 327.7[a]
11	Isocorydine*	447.1 ± 251.7	944.1 ± 267.9[a]
12	Laurotetanine*	515.6 ± 265.5[b]	580.1 ± 275.4[a]
14	*N*-methyllaurotetanine**	960.9 ± 374.9[b]	1640.1 ± 568.2[a]

Means with different superscripts (a or b) in both columns are significantly different *Tukey test ($p < .05$) **Kruskal Wallis test ($p < .05$).

Table 5
Alkaloid content in boldo leaves from trees of different ages (μg/g DW).

N	Compound	Cultivated	DBH < 40 cm		DBH ≥ 40 cm
		Saplings n = 50	MP-3 n = 15	MP-1 and – 2 n = 50	LA n = 15
1	Higenamine*	108.7 ± 89.3[b]	42.8 ± 30.2[b]	Nd	42.7 ± 53.0[b]
2	Coclaurine*	1395.9 ± 356.1[a]	1076.7 ± 472.2[b]	Nd	758.1 ± 453.4[b]
3	*N*-methylcoclaurine*	653.7 ± 358.5[a]	323.1 ± 245.4[b]	Nd	236.5 ± 197.2[b]
5	Laurolitsine*	197.2 ± 90.0[a]	125.0 ± 59.2[b,c]	158.9 ± 131.6[a,b]	177.4 ± 183.4[c]
6	Boldine*	189.5 ± 92.1[a]	109.8 ± 53.9[b]	140.7 ± 95.9[b]	107.2 ± 81.4[b]
8	Reticuline**	557.3 ± 278.9[b]	244.7 ± 177.2[a]	181.9 ± 131.7[a]	296.4 ± 244.6[a]
11	Isocorydine*	1193.7 ± 519.9[a]	756.2 ± 312.2[b]	386.9 ± 245.8[c]	617.0 ± 357.8[b]
12	Laurotetanine*	2215.4 ± 836.2[a]	464.3 ± 237.8[c]	492.6 ± 258.2[b]	633.5 ± 298.2[b]
14	*N*-methyllaurotetanine**	2367.0 ± 993.9[c]	480.2 ± 198.6[a,b]	403.7 ± 241.6[a]	1044.5 ± 636.5[b]
15	Secoboldine**	23.8 ± 20.8[b]	14.6 ± 7.1[a]	Nd	21.7 ± 10.9[a]
16	*N*-methylsecoboldine**	78.0 ± 34.2[b]	39.5 ± 15.5[a]	43.3 ± 32.9[a]	42.8 ± 26.9[a]

Results are reported as mean ± SD. Values with the same superscript in different columns are not significantly different. *Tukey test ($p < .01$). **Kruskal Wallis test ($p < .05$); nd: not determined.

Table 6
Alkaloid content of the woody components of two *P. boldus* populations of different ages (μg/g DW ± SD).

N	Compound	MP-1 + MP3		LA	
		Bark n = 30	Wood n = 30	Bark n = 15	Wood n = 15
1	Higenamine**	52.8 ± 100.5[b]	24.4 ± 35.6[b]	3.5 ± 7.0[a]	1.7 ± 1.6[a]
2	Coclaurine**	10,094.1 ± 3099.3[c]	835.9 ± 803.7[a,b]	316.5 ± 341.9[b]	1341.8 ± 838.3[a]
3	*N*-methylcoclaurine**	529.2 ± 311.5[c]	57.4 ± 48.0[b]	16.5 ± 12.1[b]	104.3 ± 45.6[a]
5	Laurolitsine*	1392.6 ± 972.6[b]	1370.7 ± 645.9[b]	643.9 ± 831.3[a]	2058.9 ± 728.5[c]
6	Boldine**	10,059.4 ± 1898.6[b]	752.3 ± 579.9[a]	1114.3 ± 1110.1[a]	1231.4 ± 832.4[a]
8	Reticuline**	3880.2 ± 2190.3[c]	316.7 ± 203.9[a,b]	255.6 ± 303.9[a]	577.6 ± 298.7[b]
11	Isocorydine **	452.9 ± 277.5[b]	16.1 ± 21.4[a]	11.8 ± 9.8[a]	21.7 ± 26.3[a]
12	Laurotetanine**	51.7 ± 64.4[a]	481.7 ± 359.8[b]	47.9 ± 98.0[a]	464.4 ± 370.2[b]
14	*N*-methyllaurotetanine**	256.2 ± 159.8[b]	8.9 ± 12.2[a]	12.7 ± 23.6[a]	10.5 ± 14.9[a]
15	Secoboldine**	452.7 ± 201.6[b]	28.0 ± 16.3[a]	34.8 ± 36.5[a]	44.2 ± 47.4[a]
16	*N*-methylsecoboldine*	284.5 ± 124.1[a]	7.5 ± 7.8[c]	35.8 ± 28.5[b]	13.5 ± 10.9[c]

Results are shown as means ± standard deviation. Average values with a common superscript in different columns are not significantly different, *Tukey test ($p < .01$); **Kruskal Wallis test ($p < .05$).

the bark and a higher one in the wood, with laurolitsine (**5**), boldine (**6**) and coclaurine (**2**) predominating in both tissues (Table 6).

It is an inescapable fact that all our analyses point to boldine contents of around 1% in the bark from branches, whereas several literature sources report values of about 5% in the trunk bark. This was commented on above as an unexpected result with the material used to isolate the major alkaloids, and the only reasonable explanation is that three decades ago boldo bark from large trees was found in commerce and that, as a consequence of excessive harvesting, this source is no longer available.

In summary, our results show the very broad variation of the alkaloid patterns of boldo leaves, bark, and wood. This variation is most noticeable between different individual trees. It is worth considering that early studies were done comparing small samples assumed to be representative of populations, whereas now we have analyzed material from individual trees, thus gaining a more reliable knowledge of the variability of this species. Based on the analysis of material from identified individuals, we can confirm that boldine (**6**) is not the major leaf alkaloid, while *N*-methyllaurotetanine (**14**) usually (but not always) is. Interestingly, *N*-methyllaurotetanine has been shown to be a moderately potent 5-HT_{1A} receptor agonist binding with $K_i = 85$ nM [57] which might be related to the purported "sedative" effect of boldo leaf infusions. Also, regarding the leaf alkaloids, yields are higher from farmed saplings than from wild-harvested material. In the latter, young, tender leaves contain significantly lower alkaloid concentrations than mature, leathery leaves, and regular harvesting seems to favor alkaloid yields. All boldo organs contain alkaloids, and even the wood has a higher concentration than the leaves. Therefore, not only the leaves and bark should be used, but also the wood, a material that is not currently extracted commercially.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Relative abundance (Table 1S) of *P. boldus* alkaloids determined by UHPLC-MS/MS and characterization of alkaloids by mass spectrometry (Table 2S) and the calibration curves, validation of the chromatographic method (Table 3S) and content of alkaloids present in the infusion of leaves and wood of *P. boldus* (Table 4S) are available as Supporting Information. Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.fitote.2018.02.020.

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