Peumus boldus Alkaloids: Quantification in Different Organs of the Tree and Assay as Tyrosinase Inhibitors

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Abstract

Sixteen alkaloids were detected in the bark, leaves, wood and root of *Peumus boldus*, including traces of secoboldine and boldine methine, not reported previously as constituents of this species. Fourteen of them were isolated and characterized spectrometrically, and the twelve most abundant ones were quantified by UHPLC/MS/MS. Boldine was dominant in the bark, laurolitsine and norisoboldine in wood and roots, and laurotetanine in leaves. Based on these findings and published biosynthetic studies their most likely biogenetic relationships were proposed. The total alkaloid mixtures from the different organs of the tree and pure isolated alkaloids were evaluated as inhibitors of tyrosinase activity. The IC_{50} values showed that the root and leaf alkaloidal extracts, and pure laurolitsine and coclaurine, most potently inhibit this activity, suggesting that they may be of interest as potential skin anti-pigmentation and/or food anti-browning agents.

Key words

Peumus boldus, Monimiaceae, alkaloids, tyrosinase inhibition.

Introduction

The *Monimiaceae* are a plant family that comprises ca. 30 genera and 200 species, which are found mainly in neotropical rain and montane/cloud forests [1]. In Chile there are three endemic species: *Laurelia sempervirens* (Ruiz & Pav.) Tul., *Peumus boldus* Mol. ('boldo' in the vernacular and internationally) and *Laureliopsis philippiana* (Looser) Schodde, the latter also present in bordering areas of Argentina [2]. *P. boldus* dominates the landscape of many parts of the central Mediterranean climate zone of Chile, especially in dry places where it shares its prominence with *Lithraea caustica* and *Quillaja saponaria* [3,4]. These trees have developed physiological mechanisms that allow them to tolerate water stress, high temperatures and strong solar irradiation [5,6], are able to regrow from the roots after felling or burning, and are appropriate to colonize land that has suffered desertification processes [7], such as reduction of tree coverage, soil erosion and lowered availability of nutrients.

A research area of growing interest during recent years is the medical use of this species based, to a large extent, on knowledge gleaned from the native pre-Columbian cultures and their present-day descendants. In this regard it is most remarkable that the aromatic boldo leaves and seaweed, both imported from different places many kilometres away, were already combined into a sort of chewing gum by some of the earliest inhabitants of South America 14,800 years ago [8]. The bark was smoked, apparently in rituals, 1,000-1,500 years ago [9], and at least at the time of the Spanish invasion (16^{th} century) the fruit was used to make a fermented beverage. More recent traditional medicinal applications include earache, headache, rheumatism, nasal congestion and, prominently, digestive and biliary

disorders [10]. Likewise, the remarkable content of polyphenols in this species [11], specially catechin [12] and relevant concentrations of phenolic alkaloids (including boldine), among other metabolites, endows it with valuable antioxidant and cytoprotective properties [10,13-17], including hepatoprotective and anti-inflammatory activities [18,19]. Boldo leaves are 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 [20]. The harvest, as carried out at present, incurs losses of hundreds of thousands of tons of shoots per year, which could be processed as a good source of secondary metabolites due to their well known content of boldine and other alkaloids. The leaves contain up to seventeen different alkaloids in modest concentrations, the characteristic boldine not being the most abundant, while the bark is considered a good industrial source of boldine and is exported as such, mainly to Europe. However, reliable quantitative analyses of boldo alkaloids in the different organs of the tree are lacking. We have now addressed this limitation, aware of the likely variability of *P. boldus*, by isolating most of these alkaloids and quantifying them in the leaves, bark, wood and roots of representative samples from Central Chile by UHPLC/MS/MS.

In recent years some aporphine alkaloids such as magnoflorine [21] and *N*-formylanonaine have been proposed for use as anti-pigmentation agents due to their low cytotoxicity [22]. More specifically, boldine is a mixed-type tyrosinase inhibitor with an estimated IC_{50} value of about 6.52 mM, binding to the free enzyme and to its complex with L-DOPA [23], an observation that prompted us to extend a similar study to the rest of the alkaloids isolated from *P. boldus* as tyrosinase inhibitors. It is important to note that melanogenesis is not a simple biochemical process (Fig. **1**). Nevertheless the activity of tyrosinase is determinant

in the overall sequence and, moreover, tyrosinase is the first and rate limiting enzyme of the global process [22]. Thus, all the compounds studied here as tyrosinase inhibitors may be considered as potential inhibitors of melanogenesis [24]. Even though the mechanism of this enzyme has not been completely elucidated, it is well known that in the presence of oxygen this oxidoreductase is able to dihydroxylate the phenolic moiety of monophenols (monophenolase activity) and also to oxidize catechols to the corresponding quinones (diphenolase activity) [25]. This dual activity makes it difficult to describe in detail the mechanistic pathways (a goal we have not addressed in this study). The use of L-DOPA as substrate simplifies the analysis since the monophenolase activity is avoided when the substrate is a catechol. In the present work, the alkaloids isolated in sufficient amounts and the total alkaloidal extracts of boldo leaves, bark, wood and roots were evaluated as inhibitors of mushroom tyrosinase following the methodology reported by Kubo *et al.* [26], where the formation of dopachrome, absorbing strongly at 475 nm [27], was monitored as indirect evidence of tyrosinase total activity.

Results and Discussion

Composition and quantification

UHPLC was used to quantify the concentrations of twelve alkaloids in different parts of the tree (Fig. **2**). The yield from each organ (based on dry weight) was 0.025, 0.035, 0.15 and 0.04 % for leaves, wood, bark and roots respectively. The most abundant alkaloid detected in the bark was boldine (around 60% of the total alkaloids). The high concentration of boldine and the much lower concentrations of other alkaloids (tetrahydroisoquinolines and aporphines) is 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. As indirect evidence, this alkaloid, at 0.01%, inhibits the feeding of *Syntomis mogadorensis* (Lepidoptera) by 50% [28], and shows a minimum inhibitory concentration of 16 mg/mL against *Candida albicans* [29].

Laurotetanine and coclaurine, at 32 and 18% respectively, were the most abundant alkaloids in the leaves. It is interesting to note that in the wood and roots both laurolitsine and norisoboldine, in almost equal concentrations, account for about 30% of the total, aside from reticuline which constitutes about 20%, but only in the roots. The presence of these secondary metabolites and their structural relationships allow us to propose a plausible complete biogenetic pathway. At this point, the biosynthetic contributions of Tewari *et al.* [30] and Schneider *et al.* [31], in addition to the *O*- and *N*-methylation sequence in the 1-benzyl-1,2,3,4-tetrahydroisoquinoline skeleton proposed by Stadler *et al.* [32,33], strongly

suggest that their formation from their common precursor (*S*)-reticuline [34] occurs as shown in Fig. **3**.

Two parallel routes follow the same sequence: ring closure to build the biphenyl system, methylation of the C-1 oxygen and demethylation of the C-2 oxygen atom of the aporphine structure. The main difference between pathways A and B resides in the fact that the latter includes an *N*-demethylation, probably prior to the formation of norisoboldine, and a final *N*-methylation to yield boldine by an alternative route from laurolitsine. These proposals agree well with what is known regarding the losses of labeled methyl groups from the oxygen atom at C-6 of reticuline (C-2 in aporphines) and from the nitrogen atom of (*S*)-[1-^{13}C,6-O^{13}CH$_3$,N-^{13}CH$_3$]reticuline and subsequent methylation of the C-2 oxygen atom to yield laurolitsine in cell suspension cultures of *P. boldus* [31]. The complete absence of any ring A catechol derivatives makes a demethylation/methylation pathway seem unlikely *en route* to the laurolitsine and boldine *O*-methylation pattern. Neither cassythicine nor its nor-analog actinodaphnine, putative 1,2-methylenedioxy precursors of boldine and laurolitsine, respectively, were detected in any of the boldo samples analyzed. Taken together with the presence of *N*-methyllaurotetanine and laurotetanine, our results suggest that the methylation/demethylation route from 1-hydroxy-2-methoxy- to 2-hydroxy-1-methoxyaporphines in boldo is more likely than or at least predominates over the alternative processes *via* catechol or methylenedioxy intermediates. Some alkaloids reported for this species: the demonstrated or likely precursors norcoclaurine and norreticuline, corydine, and the biosynthetically divergent pronuciferine, sinoacutine [35] and pallidine [36], were not found in this work. Conversely, glaucine, 6a,7-dehydroboldine, secoboldine, and *N*-methylsecoboldine (boldine methine) were detected at trace levels.

6a,7-Dehydroboldine has been suggested to be an oxidation artifact of boldine [10]. The presence of the secoaporphines is more intriguing, as the conditions in which the extractions were performed are not generally viewed as conducive to Hofmann elimination reactions. More specifically, the presence of *N*-methylsecoboldine would seem to denote the presence of the thus far undetected quaternary alkaloid *N*-methylboldinium.

In summary, we have now quantified most of the *P. boldus* alkaloids in the different parts of the tree. We are aware of the often overlooked fact that botanical species are highly variable in their content of secondary metabolites, and view our present work as a preliminary contribution using a methodology that should be followed by further studies to address this shortcoming. Nevertheless, this information should contribute to the future elucidation of metabolic pathways, product translocation and variability in this valuable medicinal plant, as well as providing a more rational basis for its industrial exploitation.

Tyrosinase inhibition

To evaluate and compare the tyrosinase inhibitory power of the alkaloidal extracts of different parts of boldo trees (Fig. **4**), inhibition values (IC_{50exp}) of all four extracts were determined prior to chromatographic purification (Table **1**). Our results show that the most active extract (leaf) is twice as potent as the wood and bark extracts. The IC_{50} values, Michaelis constants (K_m), maximum rates (V_{max}) and catalytic constants (K_i) were determined similarly for each of the pure isolated alkaloids. All these results are shown in Table **2**. It may be seen that our value for boldine, the least potent tyrosinase inhibitor in our collection, is practically the same as that found in the literature [23], while its *N*-demethylated analog, laurolitsine, is about six times as potent. Other relatively potent

constituents are the 1-benzyltetrahydroisoquinolines coclaurine and *N*-methylcoclaurine, but their IC_{50} values are still much higher than those of the reference kojic acid, arbutin, and gallic acid and only slightly better than magnoflorine [21].

In order to better understand the inhibitory activity of each part of the tree, the IC_{50} value and the relative abundance of each alkaloid were compared, as shown in Fig. **5**. Inspection of Fig. **5** suggests that the strong inhibitory activity observed for the root extract (IC_{50} = 0.20 mg/mL) is presumably due to the combined higher relative contents of laurolitsine and coclaurine, two of the most powerful inhibitors evaluated (plus possibly norisoboldine), and lower concentrations of alkaloids with weaker activity, i.e. boldine, reticuline and laurotetanine. From this figure one might imagine that the activity of root and wood extracts should be quite similar, but in fact the wood is considerably less active than the root extract (IC_{50} = 0.37 mg/mL). Finally, the even weaker inhibitory power of the bark extract seems to be given by the very high concentration of boldine, by far the poorest tyrosinase inhibitor alkaloid in the tree.

We also estimated the IC_{50est} values expected for each extract (Table **1**), considering the experimental IC_{50exp} values of the different alkaloids, weighted by their relative abundances:

$$IC_{50est} = \sum_{1}^{n} X_i \, IC_{50i}$$

where X_i is the molar fraction and IC_{50i} is the IC_{50exp} value (mM) of the ith alkaloid in an extract for which the n identified alkaloids are considered. The ratio between IC_{50exp} and

IC_{50est} allows a rough estimate of a possible synergy of the alkaloids in a mixture. Thus, any $IC_{50exp} < IC_{50est}$, i.e. a stronger effect determined experimentally for an extract than that expected from the linear combination of the potencies of the individual alkaloids in the mixture, suggests that the combined alkaloids are acting synergically. The ratios found are approximately 0.3 for leaves, bark and roots, and 0.6 for the wood indicating that synergy among the different alkaloids is likely. The somewhat less favorable ratio for the wood extract might be a reflection of its relatively high content of the strongly active laurolitsine (and presumably norisoboldine, which could not be assayed because too little was isolated). None of the three aromatized products – dehydroboldine, secoboldine and boldine methine - were evaluated as tyrosinase inhibitors because they are found in very small quantities in the plant.

To complete the kinetic study the type of inhibition for *N*-methylcoclaurine, one of the most potent inhibitors in *P. boldus,* was studied (Fig. **6**).The Michaelis constant (K_m) and maximum rate (V_{max}) were determined from the double-reciprocal plot. These kinetic parameters are in reasonable agreement with the widely diverse values reported for the same mushroom tyrosinase (EC 1.14.18.1) by different authors (Table **1S**). The inhibitory behavior exhibited by *N*-methylcoclaurine suggests that this alkaloid competes with the endogenous substrate L-DOPA for the tyrosinase active site. The phenolic structure of *N*-methylcoclaurine might also make it a substrate for the monophenolase activity of tyrosinase, thus decreasing the free enzyme available to oxidize L-DOPA (diphenolase activity) and consequently lower its apparent affinity for the substrate, without modifying the V_{max}. The inhibition constant K_i of *N*-methylcoclaurine was 0.82 mM (at 0.84 mM L-DOPA). It is important to note that the formation of dopaquinone and finally dopachrome,

due to the diphenolase activity of the enzyme, is limited by the concentration of molecular oxygen in the active site, a condition that was not controlled as the experiments were carried out under atmospheric conditions, implying that K_m and K_i are apparent constants.

Materials and Methods

Materials

Leaves, bark, wood and roots (the latter from dead trees) of *P. boldus* were collected in September 2014 near María Pinto (33°31´ S and 71°07´ W, about 200 m above sea level), Santiago Metropolitan Region, Chile. Voucher specimens, identified by Alicia Marticorena, are deposited in the Herbario CONC (herbarium of the Botany Department, University of Concepción, Concepción, Chile). Mushroom tyrosinase (EC 1.14.18.1), Triton™ X-100, silica gel 60 Å (40-63 µm) and L-DOPA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monosodium phosphate, disodium phosphate, anhydrous sodium sulfate and dimethylsulfoxide (analytical grade) were purchased from Merck (Darmstadt, Germany). All solvents used were of analytical grade or double distilled. Water was of ultrapure grade.

Extraction

Dried and ground bark, wood, roots and leaves (3.5, 10.0, 4.0 and 2.0 kg respectively) were submerged three times for 4 hours each time in MeOH (5 L/kg) at 50 °C. The liquors 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.

Isolation

Each total alkaloid extract was purified by column chromatography 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.

Reference Standards

(*S*)-Glaucine and (*S*)-predicentrine were prepared from boldine by methylation with diazomethane [37,38]. Boldine was converted into secoboldine and *N*-methylsecoboldine (boldine methine) as described in the literature [39,40]. (*RS*)-Norreticuline, *(RS)*-coclaurine and *(RS)-N*-methylcoclaurine were synthesized by the Bischler-Napieralski route [41]. *(RS)*-Norcoclaurine was prepared by demethylation of *(RS)*-coclaurine [42]. Isocorydine HCl was purchased from PhytoLab GmbH & Co. (Vestenbergsgreuth, Bavaria, Germany). Norisoboldine was purchased from AK Scientific Inc. (Union City, California, USA). Corydine was purchased from ALB Technology Limited (Hong Kong, China). Reticuline was isolated from the bark of *Cryptocarya alba* and laurotetanine was isolated from the bark of *Laurelia sempervirens*.

Liquid Chromatography and Mass spectrometry

All the samples were analyzed in an EkspertUltraLC 100-XL ultra-high pressure liquid chromatograph coupled to an electrospray (ESI) ABSciex Triple Quad 4500 (LC-MS/MS) triple quadrupole mass spectrometer. A PhenomenexSynergi™ Fusion-RP 80 Å (50 mm x 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 volume of injection 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 LC-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 0.01-1 µg/mL range, for which the following equations were obtained: boldine $y = 1.6 \times 10^8 x + 6.7 \times 10^6$, $R^2 = 0.997$; norcoclaurine $y = 3.6 \times 10^7 x - 7.9 \times 10^5$, $R^2 = 0.999$, norreticuline $y = 6.0 \times 10^7 x - 2.3 \times 10^6$, $R^2 = 0.995$, coclaurine $y = 2.3 \times 10^7 x + 6.9 \times 10^4$, $R^2 = 0.999$; laurolitsine $y = 8.4 \times 10^7 x + 9.4 \times 10^4$, $R^2 = 0.998$; isocorydine $y = 2.0 \times 10^8 x + 5.7 \times 10^6$, $R^2 = 0.998$; laurotetanine $y = 4.9 \times 10^7 x + 4.8 \times 10^2$, $R^2 = 0.998$; *N*-methylcoclaurine $y = 1.7 \times 10^7 x + 4.9 \times 10^2$, $R^2 = 0.998$; *N*-methyllaurotetanine $y = 4.1 \times 10^7 x + 6.9 \times 10^2$, $R^2 = 0.999$; norglaucine $y = 9.6 \times 10^8 x + 3.4 \times 10^6$, $R^2 = 0.997$, norisoboldine $y = 8.4 \times 10^7 x - 5.2 \times 10^1$, $R^2 = 0.999$, predicentrine $y = 2.1 \times 10^8 x + 3.3 \times 10^6$, $R^2 = 0.999$, reticuline $y = 1.6 \times 10^8 x + 6.6 \times 10^6$, $R^2 = 0.997$, *N*-methylsecoboldine $y = 1.1 \times 10^8 x + 4.2 \times 10^6$, $R^2 = 0.998$, $1{,}7 \times 10^8 + 8{,}0 \times 10^6$ $R^2 = 0.996$.

Tyrosinase inhibition (EC 1.14.18.1)

All measurements were performed in a Spectroquant® Pharo 300 Merck spectrophotometer at 25 °C following the procedure reported by Kubo *et al.* [26]. A stock solution of mushroom tyrosinase (5771 units per mL or 8.06 µM) was stored at -20 °C prior to use. All inhibitor solutions were prepared by mixing 1.8 mL of 0.1 M phosphate buffer, 0.6 mL milliQ water, 0.1 mL of sample dissolved in 1 mL of DMSO (HPLC grade, < 3.3 % v/v),

and 0.1 mL of tyrosinase stock solution, except for 6,7a-dehydroboldine, which necessitated the addition of Triton™ X-100 (1.6% v/v). This mixture was pre-incubated for 5 min at 25 °C before addition of an aliquot of a light-protected solution of L-DOPA (6.3-22.1 mM in the spectrophotometer cuvette). To validate the method, arbutin, kojic acid and gallic acid were used as positive controls (results not shown). The tyrosinase activity was evaluated following the change of absorbance at 475 nm over time, typically at ca. 10 min.

Supporting information

Comparison of kinetic data for tyrosinase and L-DOPA as substrate, chromatograms of organs and relative abundance of *P. boldus* alkaloids determined by UHPLC and characterization of alkaloids by mass spectrometry are available as Supporting Information.

Acknowledgements

Thanks are due to the Fondo de Investigación del Bosque Nativo (CONAF) for grant N° 055/2013, and Ms. Estefanía Hugo for synthesizing norreticuline.

Conflict of Interest

The authors declare no conflict of interest.

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Legends for Figures

Fig. **1**. Accepted melanogenesis pathway[44-47].

Fig. **2**. Alkaloidal composition of the crude basic extracts isolated from different parts of the tree.

Fig. **3**. Proposal of biosynthetic pathways of boldine from reticuline in P. *boldus*. Pathway A, without modification of the *N*-methyl group. Pathway B, with loss (prior) and recovery (final) of the *N*-methyl group.

Fig. **4**. Inhibition of tyrosinase activity by *P. boldus* extracts.

Table **1**. Effects on tyrosinase activity of extracts of different parts of *P. boldus* trees.

Table **2**. Effects on tyrosinase activity of commercial alkaloids and compounds isolated from *P. boldus*.

Fig. **5**. Inhibitory power versus relative abundances of alkaloids in different parts of *P. boldus.*

Fig. **6**. Kinetics of inhibition of tyrosinase activity by (*RS*)-*N*-methylcoclaurine.

Fig. **1**.

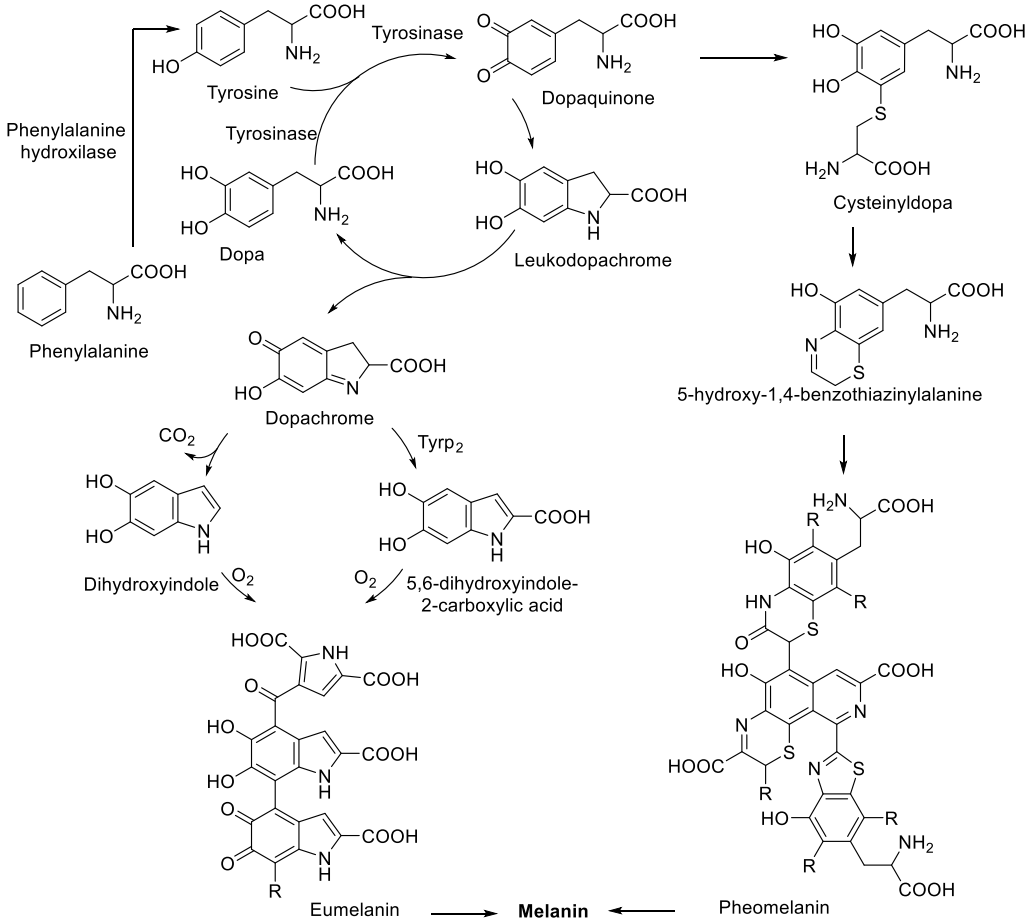
Tyrosinase
Tyrosine
Dopaquinone
Tyrosinase
Phenylalanine hydroxilase
Dopa
Leukodopachrome
Cysteinyldopa
Phenylalanine
Dopachrome
5-hydroxy-1,4-benzothiazinylalanine
CO_2
$Tyrp_2$
Dihydroxyindole
O_2
O_2
5,6-dihydroxyindole-2-carboxylic acid
Eumelanin
Melanin
Pheomelanin

Fig. **2**.

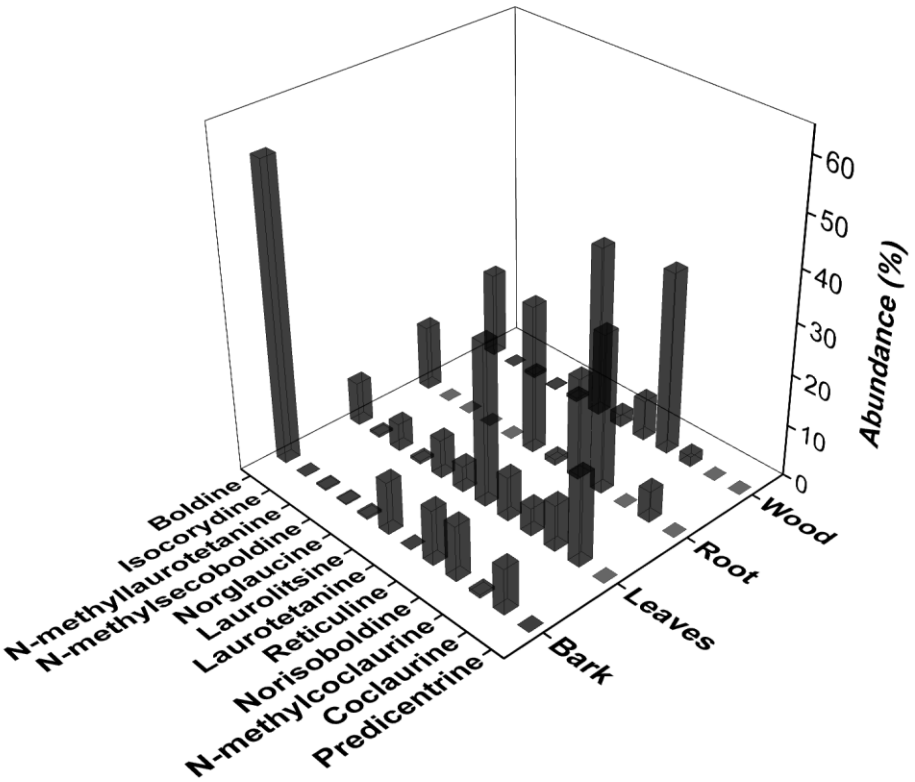
Boldine
Isocorydine
N-methyllaurotetanine
N-methylsecoboldine
Norglaucine
Laurolitsine
Laurotetanine
Reticuline
Norisoboldine
N-methylcoclaurine
Coclaurine
Predicentrine
Bark
Leaves
Root
Wood
0
10
20
30
40
50
60
Abundance (%)

Fig. **3.**

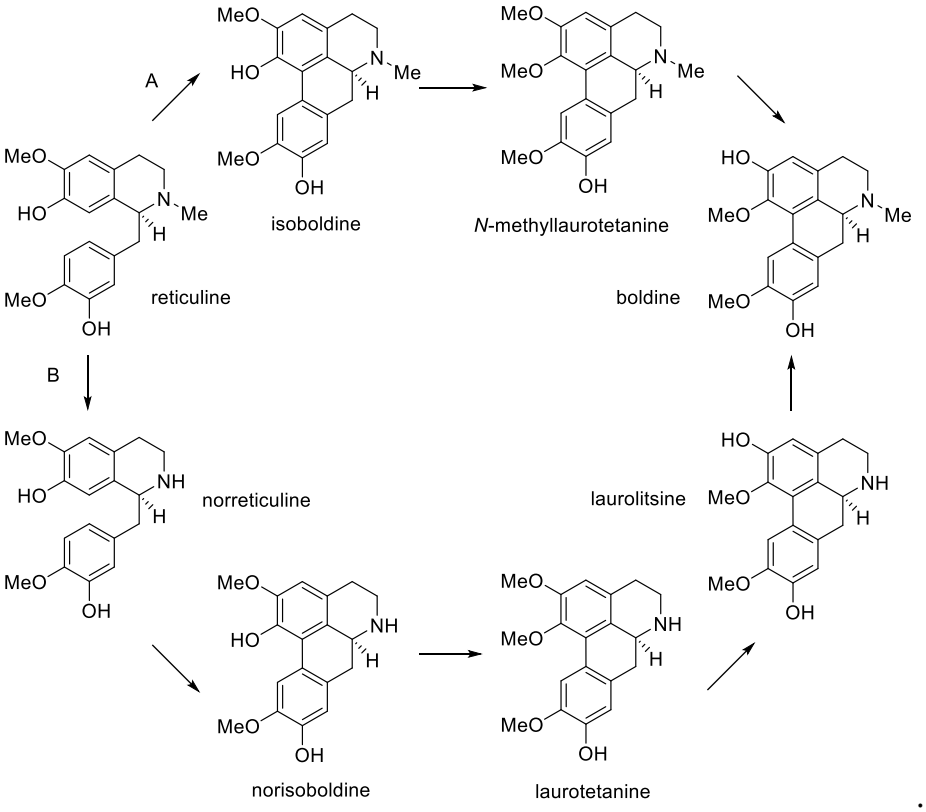
A
B
reticuline
isoboldine
N-methyllaurotetanine
boldine
norreticuline
norisoboldine
laurotetanine
laurolitsine
MeO
HO
OH
N
NH
Me
H

Fig. **4**.

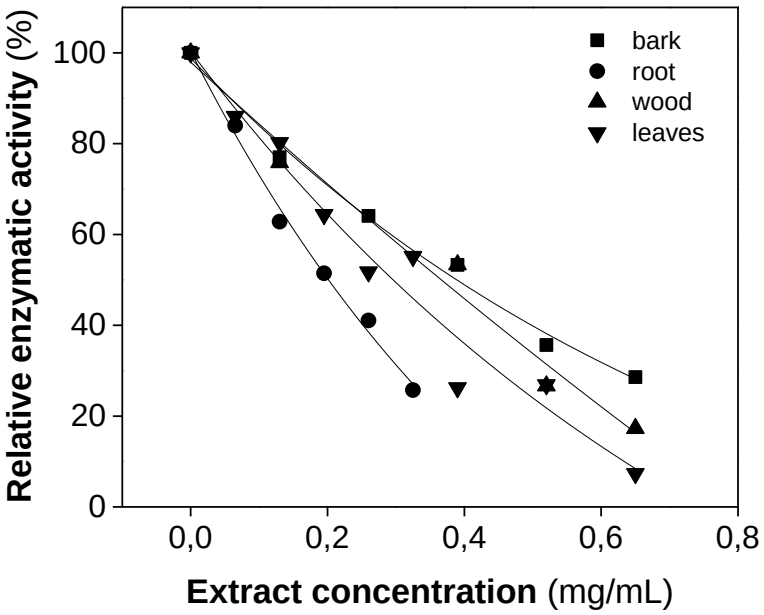
Relative enzymatic activity (%)
100
80
60
40
20
0
0,0
0,2
0,4
0,6
0,8
Extract concentration (mg/mL)
bark
root
wood
leaves

Table **1**.

	IC_{50exp} (mg/mL)[a]	IC_{50exp} (mM)[b]	IC_{50est} (mM)[c]	IC_{50exp}/ IC_{50est}
Root	0.20	0.63	2.18	0.29
Leaves	0.29	0.90	2.58	0.35
Wood	0.37	1.15	1.93	0.59
Bark	0.39	1.21	4.52	0.27

[a]obtained from Fig. **4**, [b]calculated using average molecular weight of alkaloids from Figure **5**, [c]calculated wih $\sum_1^n X_i\, IC_{50i}$

Table **2**.

Inhibitor	IC_{50} (mM)[b]	K_m (mM)[c]	V_{max} (mM/min)[c]
N-methyllaurotetanine[a]	2.23	3.58	0.11
Boldine[a]	6.56	0.33	0.03
Laurolitsine[a]	0.96	0.99	0.08
Reticuline[a]	4.71	0.45	0.04
Laurotetanine[a]	3.38	0.44	0.04
Isocorydine[b]	3.93	0.77	0.05
(*RS*)-*N*-methylcoclaurine[b]	1.46	1.10	0.04
(*RS*)-Coclaurine[b]	1.29	1.75	0.07
Gallic acid	0.200	N.D	N.D.
Arbutin	0.061	N.D	N.D.
Kojic acid	0.014	N.D	N.D.

[a]Isolated and purified from *P. boldus*; [b] Commercial or synthetic. [c]at 0.84 mM L-DOPA

Fig. **5**

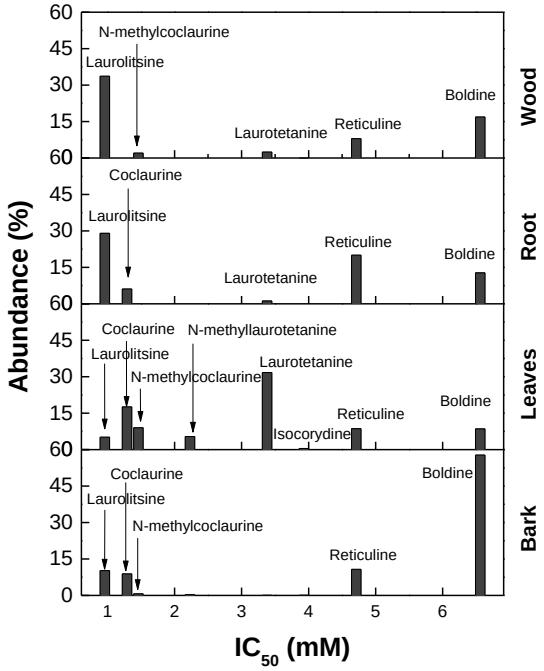
Abundance (%)
IC50 (mM)
Wood
Root
Leaves
Bark
N-methylcoclaurine
Laurolitsine
Laurotetanine
Reticuline
Boldine
Coclaurine
N-methyllaurotetanine
N-methylcoclaurine
Isocorydine

Fig. **6**