Boldine and its antioxidant or health-promoting properties

Peter O'Brien [a], Catalina Carrasco-Pozo [b], Hernán Speisky [b,c,*]

[a] *Graduate Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto, Toronto, Ont., Canada*
[b] *Micronutrients Unit, Nutrition and Food Technology Institute, University of Chile, El Líbano 5524, Macul, P.O. Box 138-11, Santiago, Chile*
[c] *Pharmacological and Toxicological Chemistry Department, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile*

Abstract

The increasing recognition of the participation of free radical-mediated oxidative events in the initiation and/or progression of cardiovascular, tumoural, inflammatory and neurodegenerative disorders, has given rise to the search for new antioxidant molecules. An important source of such molecules has been plants for which there is an ethno-cultural base for health promotion. An important example of this is boldo (*Peumus boldus* Mol.), a chilean tree whose leaves have been traditionally employed in folk medicine and is now widely recognized as a herbal remedy by a number of pharmacopoeias. Boldo leaves are rich in several aporphine-like alkaloids, of which boldine is the most abundant one. Research conducted during the early 1990s led to the discovery that boldine is one of the most potent natural antioxidants. Prompted by the latter, a large and increasing number of studies emerged, which have focused on characterizing some of the pharmacological properties that may arise from the free radical-scavenging properties of boldine. The present review attempts to exhaustively cover and discuss such studies, placing particular attention on research conducted during the last decade. Mechanistic aspects and structure–activity data are discussed. The review encompasses pharmacological actions, which arise from its antioxidant properties (e.g., cyto-protective, anti-tumour promoting, anti-inflammatory, anti-diabetic and anti-atherogenic actions), as well as those that do not seem to be associated with such activity (e.g., vasorelaxing, anti-trypanocidal, immuno- and neuro-modulator, cholagogic and/or choleretic actions). Based on the pharmacological and toxicological data now available, further research needs and recommendations are suggested to define the actual potential of boldine for its use in humans.

Keywords: Boldine; Antioxidants; Free radicals; Oxidative stress; Aporphine-like alkaloids; Boldo

Contents

* Corresponding author. Tel.: +56 2 678 1448; fax: +56 2 221 4030.
E-mail address: hspeisky@inta.cl (H. Speisky).

1. Introduction

(*S*)-2,9-Dihydroxy-1,10-dimethoxy-aporphine (Boldine) and other related aporphine alkaloids have been shown to behave as potent antioxidants in a number of experimental models. Pharmacological activities, such as cyto-protective, anti-tumour promoting, anti-inflammatory, antipyretic and antiplatelet, have been associated with the ability of boldine to scavenge highly reactive free radicals. The occurrence and chemistry of boldine and closely related congeners will first be briefly recalled. The present review will address and discuss results from studies conducted mostly over the last decade on the antioxidant activity of boldine, and when relevant, of some structurally related compounds, expanding into the main pharmacological properties, which arise from such activity. In addition, it will attempt to cover data supporting pharmacological properties of these substances, which are not necessarily related to its antioxidant activity.

1.1. Occurrence and chemistry of boldine, derivatives and congeners

Boldine (Table 1), is the major leaf and bark alkaloid of the Chilean boldo tree. The boldo tree (*Peumus boldus* Molina, Monimiaceae) grows abundantly in the more humid ecosystems of the Mediterranean climatic region of central Chile and extends into the northern half of the much rainier Chilean lake district, between 33° and 39° South latitude. The name "boldo" or "boldu" is presumably derived from the indigenous Mapuche verbs "weltum" (to sprout again) or "volitum" (to put out new roots), which may refer to this feature. The indications for the use of boldo are extremely broad in range and just as unsubstantiated. According to pharmacopoeias and treatises dealing with medicinal plants, boldo extracts have been used for the treatment of headache, earache, rheumatism, "nervous weakness", dropsy, dyspepsia, menstrual pain, urinary tract inflammation and has also been claimed to be a sedative and mild hypnotic [1,2]. Boldo leaves contain between 0.4 and 0.5% of at least 17 different alkaloids belonging to the large benzylisoquinoline-derived family [3]. Boldine is the major alkaloid as it accounts for 12–19% of the total alkaloid content [4]. Recently, Quezada et al. [5] estimated boldine content in boldo leaves as 0.14%, a value, which is similar to the 0.12% value previously reported by us [6]. Boldo leaves contain tannins, essential oils (mainly ascaridole and cineole) and flavonoids, of which catechin was recently reported to be the most abundant one [5,7]. Since the contents of catechin, gallic acid and tannic acid in boldo are much higher than that of boldine, the former compounds appear to contribute by a greater extent to the total antioxidant capacity of boldo infusions [5,7]. Boldo bark contains unusually high concentrations of boldine, with yields of up to 6% based on dry weight [8]. Besides boldine, boldo contains, although in much smaller amounts, several other structurally related alkaloids, amongst which glaucine, an *O*-dimethylated form of boldine (Table 1) and the unsaturated boldine analogue 6a,7-didehydroboldine are found [2] (Fig. 1). Amongst the non-aporphinoid alkaloids of boldo, the benzyltetrahydroisoquinoline (*R*)-

Table 1
Structure of aporphines

Name	1	2	3	6	9	10	11
Boldine	OCH_3	OH	H	CH_3	OH	OCH_3	H
Glaucine	OCH_3	OCH_3	H	CH_3	OCH_3	OCH_3	H
Isoboldine	OH	OCH_3	H	CH_3	OH	OCH_3	H
Bulbocapnine	–O–CH_2–O–		H	CH_3	H	OCH_3	OH
Anonaine	–O–CH_2–O–		H	H	H	H	H
Apomorphine	H	H	H	CH_3	H	OH	OH
3-Bromo-boldine	OCH_3	OH	Br	CH_3	OH	OCH_3	H
3-Iodo-boldine	OCH_3	OH	I	CH_3	OH	OCH_3	H
N-methylglaucinium	OCH_3	OCH_3	H	$(CH_3)_2$	OCH_3	OCH_3	H
N-methylboldinium	OCH_3	OH	H	$(CH_3)_2$	OH	OCH_3	H

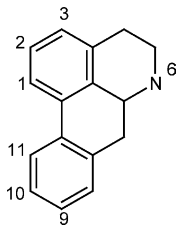
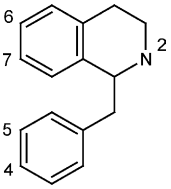


Table 2
Structure of benzylisoquinolines

Name	2	4	5	6	7	
Reticuline	CH_3	OCH_3	OH	OCH_3	OH	
Coclaurine	H	OH	H	OCH_3	OH	
Laudanosine	CH_3	OCH_3	OCH_3	OCH_3	OCH_3	
Laudanosoline	CH_3	OH	OH	OH	OH	

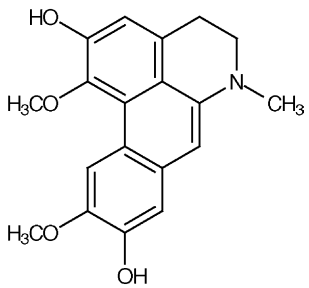


Fig. 1. 6a,7-Didehydroboldine.

reticuline (Table 2) is a common biosynthetic precursor of 1,2,9,10- and 1,2,10,11-tetraoxygenated aporphines. Boldo bark has also a considerable amount of (*R*)- and (*S*)-coclaurines (Table 2), which are common precursors of benzylisoquinoline-derived alkaloids. Although present as a minor constituent, boldine also occurs in a number of other species of the Monimiaceae, Magnoliaceae and Lauraceae, along with many other aporphinoids.

The analytical detection and quantification of boldine, early relied on the use of colorimetric, paper electrophoresis, voltammetric, thin layer and gas chromatographic methods. Based on the two absorption maxima (282 and 303 nm) of boldine, an HPLC method coupled to a UV detector was subsequently developed by Pietta et al. [9] to be applied to pharmaceutical preparations and by our laboratory [10] to assay boldine in plasma and in other biological fluids, making it possible to initiate pharmacokinetic studies. Using the latter method, we showed that upon its oral administration to rats (50–75 mg/kg) boldine was rapidly absorbed and preferentially concentrated in the liver [11].

1.2. Antioxidant activity of boldine

Research on natural antioxidants, prompted by the growing interest in free radical-induced biological damage, intensified in the 1980s and 1990s partly as a result of the perceived need to screen endangered floras for substances of potential therapeutic utility. Of particular interest as possible sources of antioxidants were medicinal plants traditionally used to treat conditions, which are related to oxidative stress, such as rheumatism and inflammatory liver diseases [12,13]. Thus, the possibilities of preventing or retarding the deleterious effects associated with excessive production of reactive oxygen species (ROS), by the use of previously unexplored groups of natural products seemed attractive as a subject of research. This endeavour was further stimulated by the unsubstantiated belief that their natural character would imply innocuousness and by increasing evidence of the expression of some forms of toxicity both, after acute and prolonged exposure to high doses of synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) [14,15]. Conversely, the ready availability of flavonoids appeared then as a potential alternative to the synthetic compounds. However, it was soon shown that some of these natural substances too might pose risks in terms of mutagenicity (vide infra) and that limitations to their in vivo use are imposed by their low oral bioavailability, unfavourable pharmacokinetics and unknown toxicity concerns [16].

Our own interest in boldine arose from the observation of structural analogies between this natural product and recognized antioxidant substances, namely structures featuring phenolic hydroxyl groups. Such interest was further prompted by the fact that nowadays boldine-containing herbal teas are widely consumed in South America and boldo leaves are being continually exported to some European countries for further pharmaceutical processing to boldine-containing concentrates. Most well-known natural and synthetic phenolic antioxidants are sterically hindered phenols, so that the free radicals generated by hydrogen abstraction from the OH group (Fig. 2, reaction B) are not only thermodynamically, but also kinetically stable or "persistent", i.e., their propensity to react with the biological substrates they protect (reaction C) is substantially lower than that of the "parent" free radical (reaction A).

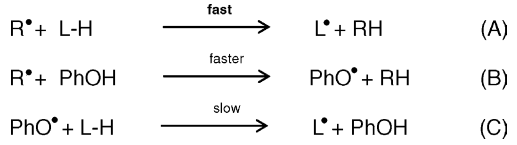


Fig. 2. Free radical (R•); biological substrate (L–H, i.e., lipid); substrate-derived free radical (L•); phenolic antioxidant (PhOH); antioxidant-derived free radical (PhO•).

However, it was our early observations of the very potent antioxidant activity of boldine in biological [17] and abiotic [18] systems that encouraged us to systematically explore its actual potential as an antioxidant and cyto-protective agent. 2,2′-Azobis[2-amidinopropane] (AAPH) is a water-soluble azo-compound whose thermal decomposition generates alkylperoxyl radicals. We showed initially that low concentrations of boldine (IC_{50} = from 5×10^{-6} to 15×10^{-6} M) effectively protected red blood cell plasma membranes against the AAPH-induced increase in antioxidant-sensitive O_2 uptake [17]. The latter parameter is considered as an index of the rate at which O_2 reacts with polyunsaturated fatty acid (PUFA)-derived free radicals, propagating the oxidation of membrane lipids. Noteworthy, in such a system the antioxidant (anti-propagation or chain-breaking) efficiency of boldine (K_i = 13 μM) was found to be similar to that reported for the widely used bioflavonoid (+)-cyanidanol-3 and about 30-fold greater than that of silybin, a mixture of lignano-flavonoids available for therapeutic use as an antioxidant [19]. Boldine was also demonstrated to effectively inhibit the spontaneous autoxidation of brain membrane lipids (assessed by production of thiobarbituric reactive substances or TBARS, O_2 uptake and chemiluminescence) with an apparent K_i of about 19–30 μM [17]. Autoxidation of lipids is a process that mainly reflects the overall rate of transition metal-dependent decomposition of lipid peroxides, both, initially occurring and those formed during the incubation conditions. Shortly before, Ríos et al. [20] observed that some phenolic benzylisoquinoline alkaloids inhibited Fe^{2+}/cysteine-induced lipid peroxidation (as TBARS production) of rat liver microsomal membranes. Later, using the same experimental model, these investigators made similar observations on a broader structural range of benzylisoquinoline alkaloids, including boldine and glaucine [21]. Subsequently, the same authors found that some of these alkaloids also protect deoxyribose against degradation induced by Fe^{3+}–EDTA in the presence of hydrogen peroxide, presumably, acting as hydroxyl radical (HO•) scavengers [22]. In fact, in studies aimed at elucidating the mechanism of the antioxidant action of boldine, we established [23] that boldine acts as a very efficient HO• scavenger. Such an ability was subsequently confirmed by Jang et al. [24] and by Youn et al. [25]. The latter investigators observed that boldine also inhibited nitric oxide production by the mitochondria. Yet, in contrast with the suggestion by Ríos et al. [20], boldine was found to either not react with superoxide radicals [23] or to react very poorly with these species [24]. The lack of superoxide-scavenging properties of boldine may be in fact regarded as a possibly advantageous feature since such radicals are physiologically generated by cells and may constitute important intra- and intercellular mediators of essential biological processes. Kringstein and Cederbaum [26] reported that boldine prevents the non-enzymatic peroxidation of microsomal lipids initiated by Fe^{2+} or the enzyme-catalyzed peroxidation initiated by Fe^{3+}–ATP with NADPH or NADH as cofactors or initiated by CCl_4 plus NADPH as cofactor. Interestingly, boldine was shown to prevent lipid peroxidation without inhibiting enzymes, such as CYP450 or its reductase or by diverting electrons away from the peroxidative process [26]. It was subsequently confirmed that boldine completely inhibited NAD(P)H/Fe^{3+}–ATP-induced human liver microsomal lipid peroxidation (K_i = 5 μM) and CYP2E1 inactivation [26]. Microsomal lipid peroxidation caused the inactivation of numerous endoplasmic reticular enzymes including CYP450. Interestingly, boldine also effectively prevented the occurrence of lipid peroxidation in microsomal membranes incubated with CCl_4 in the presence of NADPH, but failed to protect the CYP2E1 from undergoing the inactivation induced by this halogenated hydrocarbon [26]. CCl_4-mediated inactivation of CYP2E1 may, therefore, be independent of CCl_4-mediated peroxidation. CCl_4 may damage proteins via initially generated electrophilic trichloromethyl (CCl_3•) radicals. Furthermore, CYP2E1 is indeed an excellent catalyst for CCl_4 metabolism and inactivation of CYP2E1 by CCl_3• may well result from covalent binding to the protein, thereby escaping the protective effect exerted by boldine on other free radical-mediated events triggered by CCl_3•. The ability of boldine is not limited to the inhibition of the peroxidation dependent on electron transfer, as it also protects against the NAD(P)H-independent oxidation of the membranes initiated by *t*-butyl-hydroperoxide (*t*-BOOH) decomposition [23].

Recently, Kubinova et al. [27] reported that when added to mouse liver microsomes, boldine inhibited CYP1A-dependent 7-ethoxyresorufin *O*-de-ethylase and CYP3A-dependent testosterone 6-β-hydroxylase activities (enzymes which bioactivate promutagens and procarcinogens) and observed that 24 h after its addition to a mouse hepatoma Hepa-1 cell line, it stimulated

glutathione *S*-transferase activity. These findings suggest the possibility that, in addition to its free radical-scavenging activity, boldine could also protect some vital cell components not only by decreasing the metabolic activation of potentially toxic xenobiotics but also by increasing their removal. More studies are needed, however, to assess the actual chemoprotective potential of boldine to act intracellularly through apparently antioxidant-independent mechanisms.

Although many substances have been found to be effective inhibitors of microsomal lipid peroxidation, some features of the antioxidant activity of boldine may distinguish it from other natural or synthetic antioxidants commonly used in foods or as reference compounds. In fact, unlike agents, such as propyl gallate or phytic acid, which chelate iron and enhance its autoxidation, boldine was shown to inhibit iron-mediated peroxidation at concentrations 10 times lower than that of the iron catalyst [23].

In addition to lipids, proteins have been increasingly recognized as major biological targets of free radicals. In fact, their oxidative damage is regarded of importance in the aging process and in the ethiogenesis of cataracts, atherosclerosis and several inflammatory diseases. When enzymes are targeted, the oxidative modification is generally evidenced as carbonyl group formation at the protein's structure and is often associated with a loss of catalytic activity. Lysozyme is particularly, susceptible to peroxyl radical-mediated inactivation. In early studies, we demonstrated the effectiveness of boldine at protecting this enzyme against inactivation induced by AAPH-derived peroxyl radicals [17,28]. More recently, we showed that such effect is closely associated with a boldine-mediated protection against the oxidation of certain tryptophan residues by peroxyl radicals, which caused lysozyme inactivation [29]. Interestingly, the enzyme inactivation and tryptophan loss prevented by boldine were found to be mechanistically dissociable from the AAPH-induced carbonyl group formation. Thus, it would seem that the antioxidant properties of boldine comprise a protection, not only of lipids but also of protein targets, which, at least in the case of lysozyme, translates into preventing the oxidation of certain amino acids involved in the enzyme's catalytic activity.

In addition to the apparent advantages of boldine over other antioxidants in biological systems, this alkaloid has emerged as also displaying potentially useful properties in abiotic systems. Most oil- and fat-containing foods undergo oxidative rancidity in the absence of added antioxidants, both during and after foodstuff preparation. Although synthetic antioxidants have a demonstrated efficiency in preventing or retarding rancidity, their use as preservatives for human consumption has become increasingly questioned on the basis of their already mentioned alleged toxicity [14,15]. Natural antioxidants, in turn, have enjoyed an increasing recognition and popularity during the last decade. The industrial use of natural antioxidants has remained limited, however, possibly due to their often higher cost and their relatively lower activity. Regarding the food preservative potential of boldine, studies conducted by us revealed that it is particularly efficient in protecting PUFA. Boldine was found to protect fish oil against spontaneous short- and long-term O_2-dependent thermal peroxidation, acting at concentrations markedly lower than those required by other antioxidants. In fish oil, long-term (36 days, 25 °C) oxidation, boldine displays an activity similar to that of quercetin and a two to three times greater activity than α-tocopherol, BHA or BHT [18]. Boldine also showed a remarkably greater potency than the latter tested antioxidants in protecting fish oil against Fe^{2+}-induced rancidity [18]. More recently, it was also found to be more efficient than quercetin and BHT in protecting bullfrog oil against heat-induced oxidation [30] and stabilizing *n*-3-PUFA of sardine oil against thermal oxidation with an efficiency greater than that of BHA, BHT and α-tocopherol, but similar to that of quercetin [31].

Under most conditions alkoxyl- and peroxyl-free radicals play a crucial role in the initiation of heat- and metal-induced oxidation of abiotic substrates. However, in the presence of UV radiation, singlet oxygen [$O_2(^1\Delta_g)$] is a major initiating species in foodstuff deterioration. Boldine-containing boldo infusions were reported to effectively protect tryptophan molecules from undergoing $O_2(^1\Delta_g)$-dependent oxidation [32]. Subsequently, Zanocco et al. [33] found that boldine and glaucine were excellent quenchers of excited O_2, particularly, in polar solvents. Kinetic studies of the reactions between these aporphines and $O_2(^1\Delta_g)$ suggest that a charge transfer complex be formed initially between the aromatic ring and O_2 and that such complex can later either transfer the electron back, giving ground-state O_2 and the alkaloids or proceed to unidentified products [33]. Thus, the ability of boldine (and possibly other aporphines) to quench $O_2(^1\Delta_g)$ may also contribute to protect foodstuffs against oxidative deterioration.

Intensive ultraviolet radiation, in addition to affecting foodstuff, can also promote harmful effects onto the skin of humans. UV light may cause a mild skin burn, skin cell DNA damage and premature skin ageing possibly resulting in skin cancer. Since free radicals are recognized to participate in either the aetiology or the development of most UV-induced skin lesions [34],

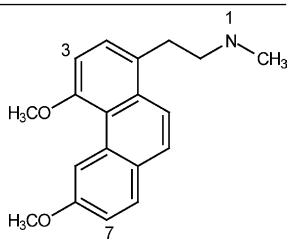
substances displaying free radical-scavenging capacity have stemmed as potentially interesting photo-protective or photo-preventive agents. Interestingly, in addition to its antioxidant properties, the boldine molecule has two major absorption peaks, at 280 and 302 nm [9,10]. The latter would confer boldine a UV light-filtering property relevant to a photo-protective action. In fact, Hidalgo et al. [35] showed boldine to be photo-unstable when irradiated at wavelengths up to 300 nm and to display a photo-protector effect against UV-B both in vitro and in vivo in mice. The photo-protection was evidenced through the prevention of the UV-induced increase in skin temperature of the rodents. More recently, Rancan et al. [36] investigated the photo-filtering properties of boldine in humans. These authors observed that the application of boldine (25 mM) onto a 12 cm^2 area of the back of volunteers protected their skin against erythema formation to an extent slightly lower than that of a commercial sun cream for which a UV-protection factor of 5 was informed. In the same study, it was observed that the in vitro irradiation of human T lymphocytes through a thin boldine-containing solution protected these cells against loss of viability with a potency even greater than that shown by octylmethoxycinnamate, a UV-B reference filter.

1.3. Antioxidant activity of boldine and of structurally related substances: structure–activity relationships

After the initial observations by Ríos et al. [20] that some phenolic benzylisoquinolines could be able to interfere peroxidative processes, several studies addressed the free radical-scavenging and/or antioxidant properties of a larger number of isoquinoline-related alkaloids. Using a chemically induced (Fe^{2+}–cysteine) microsomal lipid peroxidation assay, Martínez et al. [21] found that, amongst several aporphines tested, boldine (IC_{50} = 20 μM) displayed almost twice as much activity as isoboldine (Table 1), suggesting the importance of the presence of a hydroxyl group in the C1 position of aporphine structures. Similarly, the greater activity of the aporphine bulbocapnine relative to anonaine (IC_{50} of 12.5 μM versus 27 μM) could be explained in terms of the importance of the presence of a hydroxyl group in position the C11 (Table 1). The same authors reported that the dihydroxy-containing apomorphine molecule (Table 1) exhibited the highest activity (IC_{50} = 3.3 μM), suggesting that the presence of a catechol with hydroxyl groups at the C10 and C11 positions would be an important feature towards the high activity displayed by most aporphines, possibly endowing the formation of an *ortho*-semiquinone anion. However, the observation that glaucine, the *O*-dimethylated boldine derivative, retains most of the parent molecule's activity suggests that the presence of hydroxyl groups can add to, but is not essential for conserving the antioxidant activity of these aporphine analogues. Using the auto-oxidation of brain homogenates, Cassels et al. [28] confirmed the apomorphine molecule as the most active antioxidant amongst the tested aporphines, but the activity of boldine and glaucine suggests that *O*-methylation causes only a marginal decrease of activity. A slightly lower activity of glaucine relative to boldine was reported by Ubeda et al. [22] using the Fe^{3+}–EDTA–H_2O_2-induced deoxyribose degradation assay (hydroxyl radical-mediated). Glaucine had only half of the activity of boldine using the Fe^{2+}/H_2O_2/ascorbic acid-induced liver microsomal lipid peroxidation assay (hydroxyl radical-mediated) and the AAPH-induced loss of lysozyme activity assay [37]. The latter authors studied also the effect of halogenation (with bromo or iodine) of boldine at the C-3 position (Table 1) and observed that such modification does not affect lyzozyme protection, but it almost double the antioxidant effectiveness on the microsomal lipid peroxidation assay. The results seen in the latter assay are in line with the recognized importance of the lipophilicity in chain-breaking lipid peroxidation within membranes.

On the early structure–activity research by Martínez et al. [21], it was suggested that, in the absence of phenolic groups, aporphines could be easily oxidized to dehydro- (actually 6a,7-didehydro) and oxo- (in fact, 6-nor-7-oxo-4,5,6,6a-tetradehydro) aporphines. Thus, based on the latter, Cassels et al. [28] suggested that in non-phenolic aporphines, the benzylic C–6a–H bond may be the initial point of free radical attack and that the neighbouring nitrogen lone pair would strongly contribute to the stabilization of the intermediate radical by further extending electron delocalization. Supporting the latter, Cassels et al. [28] showed that compared to glaucine, *N*-methylglaucinium (Table 1), an analogue in which the nitrogen is protonated, is completely devoid of activity (in the lysozyme activity protection assay). Milián et al. [38] reported that *N*-methylboldinium (Table 1) is only half as active as boldine in scavenging reactive oxygen species generated by a hypoxanthine–xanthine oxidase system. Thus, the stabilization provided by the nitrogen lone pair would be essential for conserving the free radical-scavenging activity seen in both, the phenolic as well as in the non-phenolic aporphines. With regard to the importance of the piperidine ring in the aporphine structure, it seems that, at least for boldine, the presence of such ring would not be essential for manifesting such activity.

Table 3
Structure of phenanthrene

Name	1	3	7	
N-allylsecoboldine	CH_2–CH=CH_2	OH	OH	
Secoboldine	H	OH	OH	
N-methylsecoboldine	CH_3	OH	OH	
N-methylsecoboldinium	$(CH_3)_2$	OH	OH	
O-dimethylated-*N*-methyl secoboldinium	$(CH_3)_2$	OCH_3	OCH_3	

In fact, *N*-allylsecoboldine (Table 3), a phenanthrenic structural analogue of boldine in which the nitrogen atom is part of a side chain (*N*-allyl derivated), has been reported to exhibit interesting antioxidants properties [39]. Like boldine [17,23], *N*-allylsecoboldine scavenges hydroxyl and peroxyl radicals and efficiently, prevents lipid peroxidation in rat brain homogenates and erythrocyte membranes. Although, there are no studies aimed at addressing the relative activity of boldine and *N*-allylsecoboldine, Milián et al. [38] compared the ability of aporphines with that of the corresponding equivalents with a phenanthrene skeleton for scavenging ROS generated by hypoxanthine–xanthine oxidase. These investigators found that boldine is less active than any of the tested phenolic (C3–OH and C7–OH) phenanthrene analogues: secoboldine, *N*-methylsecoboldine and *N*-methylsecoboldinium (*N*-dimethylsecoboldine) (Table 3). The higher activity reported for phenolic-phenanthrenic relative to phenolic-aporphinic molecules may reside on the existence of a third benzylic ring which may confer a greater capacity to delocalize the phenoxy free radical to the former. Nonetheless, the same group observed that boldine was still more active than the non-phenolic phenanthrene, *O*-dimethylated-*N*-methylsecoboldinium (Table 3) and that the presence of free HO groups was an absolute requirement for the activity of phenanthrene derivatives.

On the other hand, in structure–activity studies conducted on benzylisoquinoline alkaloids, Martínez et al. [21] found that reticuline and laudanosine (analogues of the aporphines isoboldine and glaucine, respectively) (Table 2), showed very poor activity. However, laudanosoline (Table 2), a bicathecol molecule, was found to exhibit the greatest activity amongst all tested benzylisoquinolines (IC_{50} = 6.8 μM). Thus, it appears that *O*-methylation substantially affects the activity of benzylisoquinolines. The presence of at least one or more catechols is a common feature amongst the most active molecules within both, the the aporphines and the benzylisoquinolines. Noteworthy, laudanosoline, despite presenting two cathecols, shows only half the activity of apormorphine, which exhibits only one of such moieties. About the latter, Cassels et al. [28] postulated that the superior ability of aporphines to trap free radicals is associated with an increased spin delocalization of phenoxy radicals in the biphenyl system. The resulting benzylic free radicals, by analogy with phenoxy radicals, would presumably be better stabilized in aporphines, as compared to their benzylisoquinolines-derived counterparts, by extended conjugation across the aporphine biphenyl system.

1.4. Pharmacological properties of boldine and related substances associated with their antioxidant activity

Here we review evidence showing the ability of boldine to limit experimentally induced processes relevant to pathophysiological conditions in which free radicals and/or peroxidation products are involved as intracellular damage mediators.

Compelling in vitro and in vivo evidence implicates free radicals as major initiators and/or mediators of biochemical events leading to cell damage [13,40]. Stemming from the free radical-scavenging ability of boldine, the pursuit of its pharmacological potential has included establishing its effectiveness in protecting cells against oxidative and lytic damage. Erythrocytes are particularly susceptible to undergo oxidative stress. AAPH has been shown to induce in intact isolated erythrocytes extensive peroxidation of membrane components (e.g., lipids and proteins) and to promote structural alterations, which lead to the rupturing the erythrocyte membrane and to the subsequent leakage of hemoglobin [41]. Using a rat erythrocyte suspension, we showed that boldine cytoprotects against cell lysis induced by AAPH [42]. Boldine prevented, time- and concentration-dependently, the AAPH-induced leakage

of hemoglobin into the medium. Such cytoprotection was observed whether the antioxidant was added to the erythrocyte suspension 1 h prior to or simultaneously with the azo-compound, suggesting that boldine was neither metabolized nor oxidized during such preincubation period [42].

The membrane permeability transition of mitochondria is known as a central event in the course of a variety of toxic and oxidative forms of cell injury as well as apoptosis induced by xenobiotics or drugs [43]. Opening of the mitochondrial permeability transition pore has been shown to induce depolarization of the transmembrane potential, release of small solutes, release of Ca^{2+} and cytochrome *c*, osmotic swelling and loss of oxidative phosphorylation. The oxidation of dopamine (DA) and 6-hydroxy-dopamine (6-OH-DA) in neurons induces free radical formation, inhibition of mitochondrial respiratory chain and opening of the mitochondrial transition pore, which is inhibited by oxidant scavengers. Based on the latter, Youn et al. [25] examined the protective effect of boldine on catecholamine (DA and 6-OHDA)-induced brain mitochondria dysfunction and on PC12cell death. At concentrations (10–100 μM), which did not affect mitochondrial permeability, boldine substantially decreased the effect of catecholamine (CA) on mitochondria swelling and attenuated the alteration of mitochondrial membrane potential. At the same concentrations, boldine attenuated the CA-induced decrease in thioredoxin reductase activity and increase in thiol oxidation and at 100 μM also decreased the mitochondrial release of cytochrome *c*. Consistent with such actions, Youn et al. [25] demonstrated that boldine (10–100 μM) decreased DA-induced death in PC12 cells (as assessed by the MTT assay and caspase-3 activity). The authors [25] concluded that, since boldine effectively reacts with $HO^{\bullet}$ radicals, its ability to attenuate CA-induced damage of brain mitochondria and to decrease the DA-induced apoptosis and cell death would reside on its radical-scavenging properties. While the in vitro observations by Youn et al. [25] suggest a potential for boldine as neuroprotective agent, subsequent work by Loghin et al. [37] conducted in vivo offered no support for such contention. Thus, when injected (s.c.) in mice at a dose of 40 mg/kg, boldine was found to be ineffective in protecting against the neurotoxic action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [37]. The latter compound generates in dopaminergic neurons an active metabolite (1-methyl-4-phenyl-pyridinium, MPP^+), which inhibits the mitochondrial electron transport chain and induces a syndrome closely resembling Parkinson's disease. Since at the same dosing, boldine was effective to increase striatal levels of DOPAC and HVA and the HVA/DA ratio in the control mice [37], its failure to promote neuroprotection against MPTP reflects that the concentration of boldine at the striatum might be enough to modify catecholamine metabolism but not to counteract the oxidative stringency induced by MPP^+ at such site. Future studies aimed at assessing the neuroprotective potential of boldine should establish its property to react with MPP^+ in relevant in vitro models and should address in vivo the ability of boldine to cross the blood–brain barrier.

Early work by Lanhers et al. [44] showed that micromolar concentrations of boldine protected isolated rat hepatocytes against damage induced by *t*-BOOH. Subsequently, they showed that lower concentrations of boldine was highly effective in protecting rat liver microsomal membranes against lipid peroxidation induced by *t*-BOOH [23]. Boldine, however, did not prevent the early (within 60 s) and sudden decline (by 50%) of reduced glutathione (GSH) and the equivalent increase in oxidized glutathione (GSSG) [45] attributed to a GSH-peroxidase-catalyzed reaction with *t*-BOOH rather than a non-enzymatic reaction with *t*-BOOH-derived free radicals. In fact, the levels of both GSH and GSSG recovered to near-basal levels after 20 min, either in the presence or absence of boldine, indicating that the protective actions of boldine do not depend on the prevention of changes in GSH/GSSG concentrations. On the other hand, the delayed addition of boldine (10 or 20 min after) to cells incubated with *t*-BOOH, while effectively blocking any further increase in TBARS, totally failed to prevent the subsequent peroxide-induced loss of cell viability, and in fact, was associated with increased cell death. Thus, it is possible that the early increase in lipid peroxidation products is sufficient to either signal or assure the continuance of other events, whether mechanistically related or not, leading to cell death. Conversely, the preincubation of hepatocytes with boldine during 150 min, at which time no boldine could be detected either intra- or extracellularly, prevented lipid peroxidation and was as effective at protecting cells against the subsequent addition of *t*-BOOH as was the simultaneous addition of boldine and *t*-BOOH to cells preincubated during 150 min under control conditions. On the basis of such observations, we suggest that early exposure to boldine may trigger events resulting in cytoprotection, perhaps involving the formation of boldine-derived metabolites with similar antioxidant properties [45].

In addition to *t*-BOOH, halogenated hydrocarbons have been widely used as paradigmatic hepatotoxic agents [46]. Using carbon tetrachloride as experimental hepatotoxin in vivo, boldine has also been evaluated as a cytoprotectant. Work conducted by Lanhers et al. [44] showed that boldine prevented CCl_4-induced

hepatitis in mice. The latter authors reported that boldine, given i.p. at a dose of 10 mg/kg partially protected mice against hepatotoxicity (as assessed by an increase in plasma GPT) when administered 30 min before the halocarbon. The fact that the protection afforded by boldine was only partial (less than 50%) may be interpreted as an indication that the dose of boldine used by Lanhers et al. [44] was insufficient, even though the dose of CCl_4 used in this study (0.03 mL/kg) was far below that employed by other investigators [46]. The partial protective effect of boldine may relate to the in vitro observation by Kringstein and Cederbaum [26] that boldine protects liver microsomal membranes against CCl_4-induced lipid peroxidation but fails to protect against CYP2E1 destruction. Presumably, before the cytochrome inactivation is completed, $CCI_3^{\bullet}$ radicals under formation binds covalently to an array of macromolecules whose functions are metabolically key in defining the hepatocyte integrity. In the latter framework, a partial degree of cytoprotection would be expected. On the other hand, it is also necessary to consider the fact that for an antioxidant to protect against tissue damage induced by a (peroxidizing) xenobiotic it is necessary that both substances (or those underlying their action) should be present simultaneously, for enough time and at the same cellular and subcellular locus. It is also a requirement that the antioxidant be present at its site of action in concentrations relevant to its cyto-protective action. Such conditions can often be met in vitro, but achieving them in vivo is much more complex. Pharmacokinetic studies conducted by us on tissue boldine concentrations suggest that for the doses used in the above-referred hepatoprotection experiments, boldine might be expected to have attain potentially effective antioxidative (and possibly cyto-protective) concentrations in the liver [11]. However, it must be kept in mind that the clearance rate of boldine from plasma is extremely high. From our kinetic studies, the half-life of boldine in plasma was estimated to be around 30 min. Thus, in order to ensure the continued presence of boldine during the first 8–12 h post-administration of the hepatotoxic agent, period during which the serum enzyme levels had not increased significantly but some damaging intracellular events had been presumably already triggered, it would probably be necessary to have the antioxidant administered repeatedly. Future assessments of new boldine analogues—with structural modifications leading to longer half-lives—might merit a more detailed in vivo evaluation of their potential hepatoprotective usefulness.

ROS are involved in the cycloxygenase- and lipoxygenase-mediated conversion of arachidonic acid into pro-inflammatory intermediates and that these reactive species are also produced in substantial amounts during inflammatory phenomena in association with leucocytes infiltration [40]. Consequently, antioxidant molecules, which may interfere with ROS generation may also display anti-inflammatory properties. Using the carrageenan-induced paw oedema assay, Lanhers et al. [44] showed that the administration of a purified boldo leaf extract to rats had an anti-inflammatory effect that they could not reproduce when boldine was administered (10 or 20 mg/kg; i.p.) instead. Subsequent work by us [47], however, demonstrated that boldine is very effective (ED_{50} = 34 mg/kg; p.o.) in reducing carrageenan-induced paw oedema in guinea pigs and in preventing (60 mg/kg; p.o.) the increase in rectal temperature seen in rabbits treated with bacterial pyrogen. These anti-inflammatory and antipyretic effects of boldine were mechanistically supported by the observation that upon its addition to a rat aortal ring preparation, boldine effectively inhibited prostaglandin biosynthesis (as 6-keto-PGF). The exact mechanisms by which boldine inhibits PG synthesis and the relationship between such action and its anti-inflammatory and antipyretic effects remains to be investigated.

On the other hand, enhanced generation of reactive oxygen and nitrogen metabolites are also implicated in tissue injury observed in several inflammatory bowel diseases (IBD). Thus, antioxidants could function ameliorating and/or preventing the inflammation and cytotoxicity seen in experimental IBD models. Based on the antioxidant and anti-inflammatory properties of boldine [44,47] Gotteland et al. [48] studied in vivo the effect of boldine in a rat model of IBD in which the colonic damage was induced by the intrarectal administration of acetic acid. These investigators found that boldine, given as a single dose (100 mg/kg) intrarectally and 30 min before acetic acid, prevented oedema formation and afforded substantial protection against the macroscopic and histologic injury. The mucosa of boldine-treated animals exhibited a substantial reduction in myeloperoxidase activity, suggesting that the antioxidant lowered the extent of neutrophils infiltration into the acid-exposed tissue. Noteworthy, recently Milián et al. [38] reported that low micromolar concentration of boldine are highly effective in preventing ROS production by human PMNs induced by the bacterial chemotactic peptide, *N*-formylmethionylleucyl-phenylalanine (f-MLP). Thus, the anti-inflammatory effect of boldine seen in the IBD model [48], could relate to antioxidant actions that work, indirectly via lowering the presence of ROS-producing cells in the affected tissue and directly, via decreasing ROS production by the same cells. Future assessments of the potential usefulness of boldine in IBD, should consider

other experimental models of chronic inflammation and using also other routes of boldine administration.

Evidence supporting the hypothesis that an increased oxidative could be involved in the pathogenesis and progression of diabetic tissue damage has prompted the experimental and clinical evaluation of the potential of antioxidants in the prevention and/or treatment of diabetes. In the single study available addressing the usefulness of boldine as anti-diabetic agent, Jang et al. [24] investigated the effect of boldine in preventing the damage induced by streptozotocin (STZ) in rats. The administration of STZ to rats induces damage to pancreatic beta cells and results in diabetes by mechanisms that are not clearly understood, but that involve an increased formation of hydroxyl radicals and other ROS in association with the prior generation and/or subsequent decay of highly reactive STZ carbonium radicals [49]. Oxidative alterations within the mitochondria, believed to contribute to the dysfunctions seen in diabetes, are partially replicated in the STZ-induced diabetes model [49]. As reported by Jang et al. [24], boldine (given at a dose of 100 mg/(kg day) in drinking water during 8 weeks) significantly prevented the elevation of blood glucose levels and the weight loss induced by STZ and attenuated the elevation of carbonyls and malondialdehyde levels seen in pancreas mitochondria. The antioxidant effects of boldine, were not evident, however, for carbonyls in liver mitochondria nor for malondialdehyde in mitochondria from kidney. Interestingly, Jang et al. [24] observed that in vitro boldine inhibited ROS production by isolated liver mitochondria when treated with antimycin *c*, a respiratory inhibitor. In vivo boldine normalised the elevated Mn–SOD and GSH-peroxidase activity in mitochondria of the pancreas of STZ-treated animals. These results suggest that boldine, by suppressing the oxidative stress existing in the pancreas of STZ-treated animals, opens up the exploration of the use of boldine and its antioxidant-related analogues in the treatment of diabetes-associated free radical overproduction.

Atherosclerosis involves three processes, oxidation, inflammation and hypercholesterolemia. In view of the previously established antioxidant [17,23,28,42] and anti-inflammatory [47] properties of boldine, Santanam et al. [50] studied the in vitro effect of boldine on the oxidizability of human LDL (assessed by diene conjugate formation) and its ex vivo effect on the oxidizability of whole plasma obtained from boldine-fed and control LDL receptor-knockout ($LDLR^{-/-}$) mice. In vitro, boldine inhibited human LDL oxidation induced by copper (5 μM) in a concentration-dependent manner (0.5–2.5 μM). The authors also investigated copper-induced diene conjugate formation ex vivo in plasma obtained from LDL-deficient mice fed simultaneously a high fat atherogenic diet and boldine (p.o. 1 or 5 mg/day) during 12 weeks. Whole plasma lipids isolated from boldine-fed ($LDLR^{-/-}$) mice were considerably less susceptible to in vitro oxidation compared to that of control animals. On the same in vivo atherogenic study protocol, boldine was shown to significantly decrease the areas of the aortic atherosclerotic lesions, by 22 and 44% for the 1 and 5 mg/day 12-week dosing, respectively. The study by Santanam et al. [50] shows that repeatedly administered, boldine effectively protects $LDLR^{-/-}$ mice from developing atherosclerotic lesions. Since boldine exerted such effect without altering plasma cholesterol, triglycerides, LDL and HDL levels, its anti-atherogenic effects may well be attributed to its antioxidant properties.

Platelet activation seen in hypercholesterolemic individuals is often associated with the synthesis of pro-inflammatory cytokines and with the release of a myriad of chemokines, which contribute to the development and progression of atherosclerotic plaques. In the context of testing the potential antiplatelet usefulness of aporphine antioxidants, Teng et al. [51] found that in platelet obtained from rabbits, boldine inhibited the aggregation induced by arachidonic acid and collagen, but not that induced by platelet-activating factor (PAF), thrombin or a thromboxane analogue U46619. They concluded that the antiplatelet effect of boldine mainly resulted from its inhibition of thromboxane A_2 formation from arachidonic acid. It remains to be established whether this in vitro inhibitory platelet aggregation ability of boldine also contributed to its in vivo anti-atherogenic properties recently shown by Santanam et al. [50].

Oxidative stress is also implicated in the molecular mechanism of tumour promotion by inducing a down-regulation of cellular gap junctions (GJIC). The latter structures play a key role in the control of cell growth, development and differentiation. The down-regulation of GJIC induced by substances, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), occurs in association with an increased production of oxidants within cells. Since the latter can lead to a rapid clonal expansion of initiated cells, in vitro models of gap junctional function can be used to screen the potential anti-tumour promoting properties of an antioxidant. Hu et al. [52] tested the efficacies of boldine and its *O*-dimethylated glaucine to inhibit the TPA-induced down-regulation of GJIC in WB-F344 rat liver epithelial cells. These investigators showed that the co-incubation of these cells with TPA in the presence of these aporphines prevented the down-regulation of GJIC in a concentration-dependent manner. At 50 μM concentration each, boldine and glaucine

completely restored the gap junction. Interestingly, these antioxidants prevented TPA-induced increment in the cellular oxidative tone (measured using the dichlorofluorescin probe) and inhibited TPA-induced translocation of protein kinase C (PKC) into the membrane. The latter effects took place concomitantly with an inhibition of TPA-induced PKC-dependent hyperphosphorylation and subsequent internalization of gap junctional connexin 43 (cx43) into the plasma membrane. These results suggest that boldine and its analogue glaucine possesses anti-tumour promoting activity through their ability to interfere in TPA-induced down-regulation of GJIC.

1.5. Pharmacological properties of boldine which are not necessarily associated with its antioxidant activity

Chagas' disease (American trypanosomiasis) is caused by several strains of *Trypanosoma cruzi* and represent a permanent threat to almost 20% of the population of Latin America. Due to the toxicity of the synthetic drugs used to treat this disease (e.g., nifurtimox and benznidazole), some natural products may be seen as a possible safer alternative. Based on early work showing that several synthetic antioxidants inhibited the respiration and growth of *T. cruzi*, Morello et al. [53] looked for the trypanosidal activity of the aporphines boldine, glaucine and apomorphine. The investigators found that all three compounds completely inhibited the growth of epimastigotes of the Tulahuén strain, LQ strain and DM 28c clone of *T. cruzi* at 500 μM (with IC_{50} ranging from 80 to 120 μM, for all strains). Although much higher concentration was required (1 mM), these alkaloids were also shown to inhibit (by 25–30%) the respiration of *T. cruzi* epimastigotes in all strains. Morello et al. [53] have suggested that these aporphines might inhibit the growth of *T. cruzi* by inhibiting mitochondrial electron transport. Although the work by Morello et al. [53] opened up the possibility of exploring the usefulness of boldine and some of its analogues for the treatment of Chagas' disease.

Macrophages play an important role in host defence mechanisms. Their principal functions include the phagocytosis of foreign particles, showing the presence of antigens and the production of cytokines and radical species. A variety of plant-derived materials can stimulate the immune system. Moreira et al. [54], who investigated the effect of boldine on mouse peritoneal macrophage functions by the liberation of H_2O_2, found that boldine (6 mM) presented a very low modulatory activity on the immune system, so the alkaloid very poorly enhanced the macrophage peroxide liberation. The very high boldine concentration employed by the latter investigators limits, however, any interpretation of their results. Using notably lower concentrations (10–30 μM), earlier work by González-Cabello et al. [55] suggested that boldine could exert in vitro an immumodulating effect on natural killer cells from patients presenting low activity and decreased blastogenesis in both, normal individuals and in patients with chronic lymphocytic leukemia. Finally, in a latter work by Philipov et al. [56], it was found that boldine inhibit the in vitro concanavalin A-induced proliferation of mouse splenocytes. Although these works suggest some possible immunomodulating properties of boldine, the actual potential of this aporphine to favourably modify cellular immune functions require confirmation and further assessment.

Several studies addressing the effects of boldine on the smooth muscle have pointed out the possibility that this alkaloid may exert some muscle-relaxing effects, which could be of potential pharmacological interest. Thus, boldine was early shown to inhibit the intestinal smooth muscle activity in the anaesthetized cat [57] and to prolong the intestinal transit in mice [58]. Working in a rat ileum preparation, our laboratory observed later that boldine exerts a concentration-dependent relaxation effect by directly interfering with the cholinergic mechanism associated with the contraction [59]. Related to the latter, we also observed that the administration of a boldine-containing boldo-dried extract to healthy volunteers significantly prolongs the oro-cecal intestinal transit rate [6]. The extent to which, in addition to boldine, some of the abundant flavonoids present in boldo may have contributed to this effect cannot be excluded yet. In turn, the cholagogic and/or choleretic effects of (boldine-containing) boldo extracts and other preparations [1], seem to also occur upon administration of pure boldine to relevant animal models [60–62]. Thus, when given by gavage to dogs (2 mg/10 kg), boldine was reported to almost double the total content of solid in bile, without modifying the biliary volume [60]. However, Lévy-Appert-Collin and Lévy [61] reported that the direct application of boldine into the duodenum of mice, at a dose of 50 or 100 mg/kg, incremented the biliary flux, by 15 and 60%, respectively. Although the flavonoid glycosides of boldo have been found to be devoided of choleretic activity by themselves, these compounds have been claimed to enhance the effect of the alkaloids [61].

In addition to an intestinal-relaxing action, boldine has also been reported to induce relaxation in previously contracted (acetylcholine-induced) smooth muscle of rat uterus [63] and to block the neuromuscular action in a muscle phrenic-nerve mouse diaphragm preparation, by

direct interaction with the post-synaptic acetylcholine receptor [64]. The muscle-relaxing effect of boldine (IC_{50} of 13.5 μM) was found to be reversible and concentration-dependent and was suggested to be mostly an action on nicotinic ACh receptors [64]. Hue et al. [65], working on the cockroach ganglion, observed that boldine acted as a specific nicotinic antagonist of the insect CNS, without displaying effects on muscarinic and GABAergic receptors. Consistent with the latter, Chuliá et al. [66] showed that boldine antagonized the α1-adrenoceptor in the guinea pig aorta, but had no effect on acetylcholine-induced contraction of the trachea. Thus, data suggest that boldine had higher specificity towards the nicotinic compared to the muscarinic receptor.

In addition to the its above described actions, boldine has been shown to block Ca^{2+} channels in rat uterus [63], rat aorta [67] and rat cerebral cortex [68], possibly through the benzothiazepine receptor site, but with an affinity considerably lower (between 1 and 2 orders of magnitude) than diltiazem. More recently, Eltze et al. [69] confirmed the Ca^{2+} channel antagonist property of boldine in a rat perfused kidney preparation, showing that boldine effectively blocks the vasoconstriction caused by elevated extracellular potassium. When tested on mouse diaphragm and in isolated sarcoplasmic reticulum membrane vesicles, boldine was found to act by inducing the release of Ca^{2+} from internal Ca^{2+} storage sites of skeletal muscle [70]. Besides its Ca^{2+} channel blocking activity, boldine was also able to antagonize α1-adrenoceptors in rat aorta [67], in guinea pig aorta [66] and in rat cerebral cortex preparations [68]. Binding competition studies conducted with [3H]-prazosin in rat cortex, indicated that boldine exhibited around 65-fold greater affinity for the native (high affinity) subtype A receptor ($pK_i = 8.31$) compared to the subtype B receptor ($pK_i = 6.50$) [71]. Consistent with the latter, Eltze et al. [69] found that boldine has an affinity approximately 25- and 15-fold higher for the subtype A compared to the subtypes B and D α-adrenoceptors, respectively. Boldine was also found not to discriminate between the α1-adrenoceptor subtypes B and D and α2-adrenoceptor subtypes A–C, at which the drug consistently displays micromolar affinity.

As a close structural congener of the paradigmatic dopaminergic agonist (*R*)-apomorphine, boldine has also been reported to exert some inhibitory effects at the central nervous system. These include neuroleptic-like, anticonvulsivant and antinociceptive actions, which may probably be mediated through the blocking of dopamine D_2 receptors [72]. Subsequent in vivo studies indicate that boldine presents in vitro good binding affinities for the D_1- and D_2-like receptors [73,74]. However, in vivo (i.p. 40 mg/kg), boldine was unable to displace [3H]-raclopride, a selective D_2-ligand, from either mice striatium or olfactory bulbs; boldine only marginally displaced [3H]-SCH23390, a selective D_1-ligand, from mice striatum [73]. Given at the same dose, boldine did not modify the apormorphine-elicited climbing, sniffing and grooming behaviours. However, in the apomorphine-induced rat yawning and penile erection model (associated with D_2-receptor), boldine inhibited both behaviours by more than 50% but did not affect striatum dopamine metabolism [73]. Therefore, although boldine may act as a dopamine antagonist, its apparently poor access to at least to certain regions of the CNS [73], added to its very short plasma half-life [11], do not allow this property to be easily revealed in some in vivo experiments.

1.6. Toxicological studies and safety concerns on boldine

The precedent of the prolonged tradition of pharmaceutical use of boldine and boldine-containing boldo preparations suggests that boldine exhibits low toxicity. In fact, relatively high doses are needed to induce side effects, toxicity or lethality in several mammalian species. Early studies by Kreitmair [62] reported that 500 and 1000 mg/kg (p.o.) were required to induce the death of mice and guinea pigs, respectively. The latter authors showed that considerably lower doses, 250 and 50 mg/kg (i.v.), were required to induce the death of mice and guinea pigs, respectively, whereas 25 mg/kg (i.v.) were required to induce the death of cats [62]. Studies conducted later by Lévy-Appert-Collin and Lévy [61], estimated an LD_{50} of 250 mg/kg (i.p.) in mice. Most animals employed in the above studies were reported to die by respiratory failure.

Studies conducted by Moreno et al. [75] reported that boldine has no mutagenicity in the SOS chromotest and in several Ames tester strains, with or without prior metabolic activation. Boldine was not able to induce point and frameshift mutations in haploid *Saccharomyces cerevisiae* cells [75]. Subsequently, Tavares and Takahashi [76] reported that boldine did not induce a statistically significant increase in the frequency of chromosome aberrations or sister chromatid exchanges in vitro in human peripheral blood lymphocytes (up to 40 μg/mL) or in vivo, in mouse bone narrow cells (up to 900 mg/kg, administrated p.o.).

Regarding the toxicity of boldine in pregnancy, Almeida et al. [77] observed that its acute administration to rats during their early pregnancy phase induced no

foetus resorption and no foetal malformation when given p.o. at 500 mg/kg. A weak but significant abortive and teratogenic effect was evident, however, at 800 mg/kg. Studying the effects of long-term administration, the same authors observed a low degree of hepatotoxicity, assessed by blood transaminases or urea levels, in rats given boldine p.o. daily at 800 mg/kg for 30 and 60 days but not seen at 500 mg/kg. No hepatic histological modifications were observed at a dose of 800 mg/kg administered for 90 days [77].

Recently, a case of anaphylactic reaction alter the intake of a boldo infusion was reported in a 30-year-old man with personal history of allergic rhino-conjunctivitis [78]. The authors suggest that a type I IgE-mediated immunologic mechanism as responsible for the patient's anaphylactic symptoms. It is not clear, however, whether such a reaction can be attributed to boldine. In fact, IgE symptoms have also been observed with other boldine-free infusions like coffee, cacao and tea. Finally, also in a recently reported case, a several fold increase in blood transaminases was detected in an elderly male patient with fatty liver who was taking daily herbal laxatives, which contained boldo leaf extracts. Transaminases returned to normal following withdrawal of the laxative [79].

2. Final remarks and future research needs

Research conducted mostly over the last decade, clearly substantiates the ability of boldine to act as a potent free radical-scavenger and antioxidant molecule. As reviewed here, ample evidence shows that boldine can react with high efficiency towards a broad scope of reactive species to either prevent or retard

Table 4
Summary of studies on the free radical-scavenging and antioxidant properties of boldine

Free radical generator/oxidant system	Oxidation substrate	Parameter measured	References
AAPH peroxyl	Red blood cell plasma membranes	O_2 uptake	[17]
	Erythrocyte suspension	Haemoglobin leakage	[42]
Autoxidation	Brain homogenate	O_2 uptake	[17]
		Chemiluminescence/TBARS	[17,28]
t-BOOH	Liver microsomal membranes	TBARS	[23]
	Isolated hepatocytes	TBARS/LDH leakage	[44,45]
		Cell viability (trypan blue exclusion)	[45]
CCl_4/NADPH	Liver microsomal membranes	TBARS	[23,26]
Fe^{2+}/cysteine	Liver microsomal membranes	TBARS	[21,23]
NAD(P)H/Fe^{3+}–ATP	Liver microsomal membranes	O_2 uptake	[23]
Fe^{2+} alone or NAD(P)H/Fe^{3+}–ATP	Human liver microsomal membranes	TBARS	[26]
AAPH peroxyl	Lysozyme	Tryptophan-associated fluorescence carbonyl groups	[29]
		Enzyme activity	[17,28,29,37]
Fe^{3+}–EDTA–H_2O_2	Deoxyribose	TBARS	[22]
Fe^{3+}–EDTA–H_2O_2–ascorbate	Deoxyribose	TBARS	[24,25]
Catecholamine-induced	Brain mitochondria	Mitochondrial permeability, swelling, membrane potential and cytochrome *c* release, thiol oxidation	[25]
Catecholamine-induced	PC12 cells	Cell viability (MTT) apoptosis (caspase-3 activity)	[25]
Fe^{2+}-induced oxidation	Fish oil	Peroxide content/TBARS	[18]
Heat-induced oxidation	Fish oil	Peroxide content/TBARS	[18]
	Bullfrog oil	Rancimat induction time	[30]
	Sardine oil	Rancimat induction time	[31]
UV-irradiation	Human T lymphocytes	Cell viability	[36]
UV-generated singlet oxygen	Tryptophan	Boldine oxidation products	[33]
Hypoxanthine–xanthine oxidase	Luminol	Chemiluminescence	[38]

the targeted oxidation of lipids, proteins and nucleic acids. Structure–activity studies have provided valuable insights into the main structural features underlying the antioxidant properties of boldine. Boldine has been broadly shown to exert potent cyto-protective effects in models of oxidative stress-induced damage. In vitro, for instance, it was able to protect isolated red blood cells, hepatocytes and neurons from undergoing cell lysis. In vivo, boldine has been found to prevent or largely ameliorate the oxidative damage and the cell injury induced to the pancreas and to the colon epithelium by oxidants in rat models of diabetes and ulcerative colitis, respectively. A large number of experimental studies have proven the effectiveness of boldine in preventing various oxidative stress-related pharmacological effects including anti-inflammatory, antipyretic, anti-tumour promoting, anti-platelet and anti-atherogenic effects. In view of the increasing recognition of the participation of free radical-mediated oxidative events in the ethiogenesis of various cardiovascular, tumoural, inflammatory and neurodegenerative pathologies, it would seem that, at this point in time, the major prospects of pharmacological application of boldine would stem from its ability to prevent oxidative stress. However, as reviewed here, a number of additional studies have shown that boldine can also promote some pharmacological effects which do not appear to be associated with its antioxidant properties, to mention are the muscle-relaxing, choleretic and/or cholagogic, anti-trypanocidal and immuno- and neuromodulating effects of boldine. Future studies on the latter actions of boldine could well broaden its potential pharmacological scope. Data available on its toxicity, which has been conducted mostly in isolated cells and in various animal models, points to a relatively low toxicity of boldine. However, its actual innocuousness in humans still remains to be established. A major limitation to assess the latter, however, would reside on the current lack of information on the actual doses of boldine that would be

Table 5
Summary of pharmacological actions of boldine associated with its antioxidant activity

Pharmacological action	Model	Effect	References
Anti-inflammatory	Carrageenan-induced paw oedema in guinea pigs. Oral administration of boldine	Effective reduction of oedema	[47]
	Acetic acid-induced colonic damage in rats. Intrarectal administration of boldine	Prevention of oedema, tissue damage and neutrophil infiltration	[47]
	f-MLP-induced ROS production by isolated human PMNs	In vitro prevention of ROS production by human PMNs	[38]
	Isolated rat aorta rings	In vitro inhibition of prostaglandin biosynthesis (6-keto-PGF)	[47]
Antipyretic	Bacterial pyrogen-induced hyperthermia in rabbits. Oral administration of boldine	Prevention of hyperthermia	[47]
Antidiabetic	STZ-induced pancreatic (beta cells) damage in rats. Oral administration of boldine	Prevention of hyperglycaemia and weight loss Reduction of TBARS and carbonyl levels in various tissues Normalization of mitochondrial antioxidant enzymes	[24]
Antiatherogenic	Copper-induced LDL oxidation in vitro	Inhibition of the oxidation of isolated human LDL	[50]
	Diet-induced atherosclerotic damage in LDLR$^{-/-}$ mice. Oral administration of boldine	Inhibition of ex vivo plasma oxidation and reduction of atherosclerotic aorta lesions	[50]
Antiplatelet	Platelet aggregation induced in vitro by arachidonic acid and collagen	Inhibition of aggregation	[25]
Anti-tumour promoting	TPA-induced down-regulation of GJIC in WB-F344 rat liver epithelial cells	Inhibition of TPA-induced down-regulation Prevention of TPA-induced increase in cellular oxidative tone and in PKC translocation	[52]
Photo-protection	UV-irradiated mice skin	Prevention of skin rise temperature	[35]
Photo-protection	UV-irradiated human back skin	Prevention of skin erythema formation	[36]
Hepatoprotection	CCl_4-induced hepatotoxicity in rats. Intraperitoneal administration of boldine	Prevention of liver damage (plasma GPT)	[44]

required to exert in humans some of the pharmacological actions reported so far and demonstrated solely in animals.

The effectiveness of boldine as an antioxidant and its relatively low toxicity observed in animals now justify the pursuit of studies aimed to explore its actual therapeutic value in phase 1 clinical studies including assessing its pharmacokinetics and major biotransformation pathways. Such studies, to be conducted in healthy subject volunteers, should establish what doses of boldine are needed to reach plasma concentrations comparable to those required for its antioxidant action. Upon establishing the latter, it would be necessary to also corroborate whether the selected doses are indeed therapeutically effective in preventing the development of the oxidative damage associated with various pathologies. These studies should be complemented by further work pursuing the characterization of both the pharmacokinetics and antioxidant effects associated with the repeated administration of boldine. The latter are also necessary in order to be sure there are no side or non desirable effects of boldine in humans (Tables 4 and 5).

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