Inhibitory Effect of Boldine on Fish Oil Oxidation

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The antioxidative effect of boldine, an alkaloid extracted from *Peumus boldus* Mol. (boldo), was assayed on the spontaneous and on the metal-induced oxidation of fish oil. The inhibitory effect of boldine was compared to those of dl-α tocopherol, the flavonoid quercetin and the synthetic antioxidants butylated hydroxytoluene and butylated hydroxyanisole. Boldine, in all assays, showed a good antioxidative effect, which was comparable to that of quercetin and even better than that of dl-α tocopherol and the synthetic antioxidants. Additive effects were observed when mixtures of boldine and quercetin or dl-α tocopherol were assayed. The present study supports the potential use of boldine as a novel natural antioxidant for fish oil.

KEY WORDS: Boldine, boldine as antioxidant, fish oil oxidation, natural antioxidants.

Boldine [(S)-2,9-dihydroxy-1,10-dimethoxyaporphine, Fig. 1A] is the most abundant alkaloid present in the leaves and bark of boldo (*Peumus boldus* Mol.), a widely distributed native tree of Chile (1). Aqueous infusions of boldo leaves containing boldine have long been used in popular medicine for their purported choleretic, diuretic, sedative and digestive stimulant properties (2). We have shown recently that boldine also presents strong antioxidant activities when tested against either the spontaneous or the chemically-induced peroxidation of biological membranes (3).

Fish oil, a major by-product of the manufacture of fish meal (4), is very rich in n-3 polyunsaturated fatty acids (5). The increasing interest in the nutritional and pharmacological properties of marine fish oils (6) has given rise to various efforts to improve the chemical and organoleptic characteristics of these products (7). Due to the high degree of unsaturation of its fatty acids, untreated fish oil is unstable and is rapidly oxidized to form toxic products (*e.g.* aldehydes, peroxides), which limit its usefulness for human consumption. Efforts directed toward stabilizing fish oil against autoxidation have involved the use of synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), and of tocopherol-like natural substances. Nevertheless, while the use of BHT and BHA for human consumption has been restricted recently (8), the use of tocopherols fails to provide effective protection against peroxidation, especially when the oil is contaminated with trace amounts of metals (*e.g.* Fe^{2+} or Cu^{2+}) (9).

Work conducted in our laboratory to stabilize fish oil against autoxidation has focused on the use of different natural products with potential antioxidant properties. We recently reported a good degree of protection against fish oil oxidation by the use of the flavonoid quercetin (10). In the present work we have tested the ability of boldine to act as an antioxidant in both spontaneous and metal-induced fish oil oxidation. We have also compared the antioxidant effect of boldine with those of quercetin and other established antioxidants when added both alone or in combination.

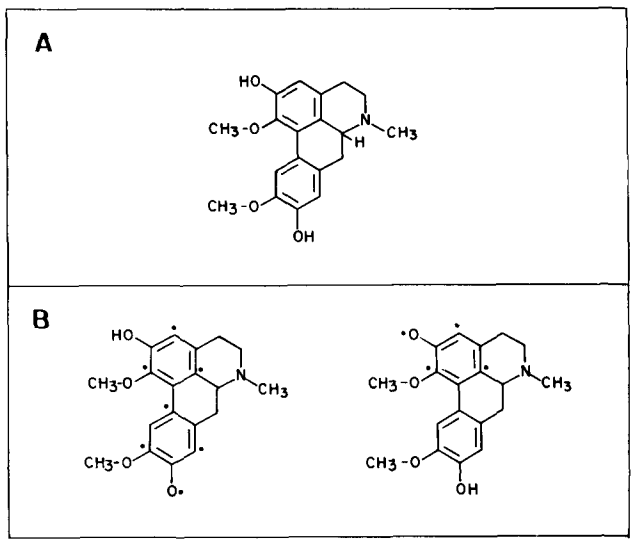

FIG. 1. A: Chemical structure of boldine. B: Alternative sites for electron delocalization in the boldine free radicals.

MATERIALS AND METHODS

Fish oil (sardine oil) obtained partially refined from a local meal factory (Corpesca S.A., Mejillones, Chile) with approximately 30% of n-3 fatty acids, was winterized and subjected to high-vacuum distillation as described before (11). The distilled oil, containing less than 2 meq peroxide/kg, was kept in the dark under nitrogen at 4°C until use in the stabilization studies.

In the short-term stabilization studies (up to 48 hr), 20-mL aliquots of oil were placed in 80 × 15-mm Petri dishes and incubated at 60°C in the dark under air. In the long-term studies, 250-mL sealed glass bottles containing 100 mL of oil were maintained in the dark at 25°C during 36 days. Oil oxidation was monitored as peroxide content [expressed as meq/kg oil according to AOAC (12)], and by the assessment of thiobarbituric acid-reactive substances (TBARS) as described by Fee and Teitelbaum (13).

According to the experiment conducted, sufficient amounts of boldine, quercetin, dl-α tocopherol, BHT, BHA, and mixtures of these substances were dissolved in 10 μL of ethanol (99.5% v/v) per gram of oil. The final concentrations are detailed either in the text or in the legends of each figure (Results section). Stabilization studies in the presence of a catalyst were performed at

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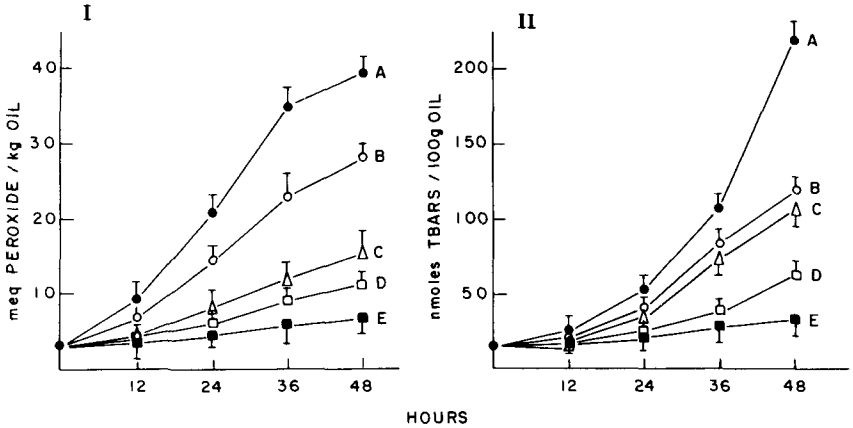

FIG. 2. Inhibitory effect of different concentrations of boldine, compared to dl-α tocopherol, on the spontaneous oxidation of fish oil. I: peroxide content, and II: TBARS. A: control, B: boldine (0.2 g/kg oil), C: dl-α tocopherol (0.8 g/kg oil), D: boldine (0.4 g/kg oil), E: boldine (0.8 g/kg oil). Other experimental conditions are described in the text.

25°C by adding $FeCl_2$ (as an ethanolic solution) to a final concentration of 250 μM.

Boldine was extracted from the bark of *Peumus boldus* Mol. and crystallized from chloroform (3). The alkaloid was chromatographically pure (thin-layer chromatography—TLC), and its identity was established by infrared (IR) and nuclear magnetic resonance (NMR) spectrometry. Quercetin, BHT and BHA were purchased from Sigma (St. Louis, MO), and dl-α tocopherol (99.5%) was a donation from Productos Roche (Santiago, Chile). All experiments were performed in quintuplicate. The results were expressed as means ± S.D., and the significance of differences between mean values was assessed by Student's t-test.

RESULTS AND DISCUSSION

As shown in Figure 2 (I and II), boldine markedly inhibited the spontaneous oxidation of fish oil incubated for 12-48 hr at 60°C. This stabilizing effect of boldine is concentration-dependent, and in seen regardless of whether the oxidation of the oil is monitored by means of the total peroxide content (Fig. 2, I) or as the accumulation of TBARS (Fig. 2, II). Compared with dl-α tocopherol, boldine exerted a similar (Fig. 2, I) or an even greater (Fig. 2, II) degree of protection at concentrations only half (0.4 g/kg) those of dl-α tocopherol (0.8 g/kg).

Results presented in Figure 3 compare the antioxidant activities of boldine, quercetin, dl-α tocopherol and of combinations of boldine with these agents with the antioxidant activities of BHT and BHA. In these protection studies, fish oil oxidation (expressed as percentage of oxidation of controls) was assessed as peroxide content of fish oil incubated at 25°C for 36 days, a period after which a maximum peroxide level is attained. Added at similar concentrations (0.8 g/kg), boldine displayed two to three times the antioxidant activity of dl-α tocopherol, BHT or BHA (Fig. 3). Boldine also showed an antioxidant activity similar to that of quercetin, the latter reported recently to be a good antioxidant for fish oil (10). Mixtures of

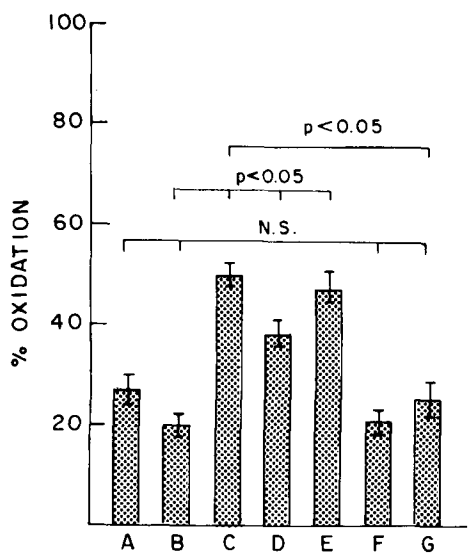

FIG. 3. Antioxidative effects of boldine and of mixtures of boldine with other antioxidants against fish oil oxidation after 36 days of incubation. A: quercetin (0.8 g/kg oil), B: boldine (0.8 g/kg oil), C: dl-α tocopherol (0.8 g/kg oil), D: BHT (0.8 g/kg oil), E: BHA (0.8 g/kg oil), F: boldine (0.4 g/kg oil) + quercetin (0.4 g/kg oil), G: boldine (0.4 g/kg oil) + dl-α tocopherol (0.4 g/kg oil). NS = not significant. Other experimental conditions are described in the text.

boldine (0.4 g/kg) and quercetin (0.4 g/kg) or dl-α tocopherol (0.4 g/kg) showed additive effects (Fig. 3). Both combinations were equally effective.

Boldine also prevented Fe^{2+}-induced (250 μM) oxidation of fish oil incubated at 25°C for up to 24 hr (Fig. 4). In fact, at a concentration of 0.8 g/kg, boldine exerted an antioxidant effect substantially greater than those of dl-α tocopherol and BHT. Of the substances tested, only quercetin showed an antioxidant effect similar to that of boldine.

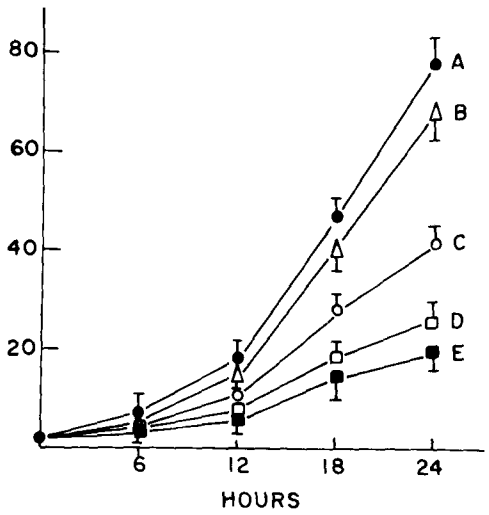

FIG. 4. Antioxidative effects of boldine and other antioxidants against fish oil oxidation induced by Fe^{2+} (250 μM). A: control, B: dl-α tocopherol (0.8 g/kg oil), C: BHT (0.8 g/kg oil), D: quercetin (0.8 g/kg oil), E: boldine (0.8 g/kg oil). Other experimental conditions are described in the text.

Our results demonstrate the ability of boldine to stabilize fish oil against both spontaneous and metal-induced oxidation. The antioxidant effect of boldine is not limited to short-term, high-thermic stringency conditions (up to 48 hr at 60°C), but also persisted even when the fish oil was stored (25°C) for up to 36 days. These studies also show that, under the experimental conditions used here, boldine is a better antioxidant than BHT, BHA or dl-α tocopherol. Noteworthy, while dl-α tocopherol fails to provide significant protection against iron-catalyzed oxidation, boldine is highly effective.

The antioxidant effects of boldine against fish oil oxidation are in line with our recent findings that this substance effectively protects biological membranes from free radical-mediated peroxidative damage (3). The antioxidant activity of boldine may be ascribed to the presence of two phenolic groups in its structure, each with an ortho substituent (see Fig. 1A). A similar chemical feature is often found in other compounds with varying degrees of antioxidant activity (14). Figure 1B shows the possible alternative sites for electron delocalization in the boldine free radicals. Together with the predictable resonance stabilization and steric hindrance of the boldine radicals, the fairly lipophilic character of the molecule makes it an interesting substance for potential use on fish oil stabilization.

We have shown that boldine, added as a single species or mixed with either quercetin or dl-α tocopherol, is effective as a stabilizer of fish oil against spontaneous or metal-induced oxidation. Our results indicate that, like the flavonoid quercetin (10), boldine could also be used as a natural antioxidant that might replace those synthetic antioxidants whose use has been questioned due to possible undesirable side effects (15). It is important to remember that BHT is no longer considered to be a generally recognized as safe (GRAS) substance by the Food and Drug Administration, USA (FDA), and it seems likely that some other synthetic compounds related to BHT such as BHA, for example, may have a similar fate. Although boldine has revealed essentially no toxicity when assayed in experimental animals (2), further physicochemical and toxicological evaluations are required to assess the effectiveness and the future of this natural compound as an antioxidant for fish oil and possible for other oils rich in polyunsaturated fatty acids.

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