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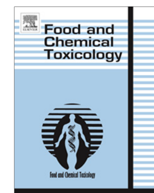

Canned bluefin tuna, an in vitro cardioprotective functional food potentially safer than commercial fish oil based pharmaceutical formulations

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ABSTRACT

Commercial canned fish species typical in the Italian market were evaluated for their lipid profile. Bluefin tuna samples showed the highest content in omega-3 fatty acids (*n*-3 PUFA) among the canned fish samples analyzed. Tests on H9C2 cardiomyocytes revealed that bluefin tuna *n*-3 PUFA may responsible for a significant cell protection against both physiological and doxorubicin-induced oxidative stress. Analogous tests performed by incubating cardiac cells with *n*-3 PUFA ethyl esters, of which most of fish oil pharmaceutical formulations (FOPF) are based, showed cytotoxicity at high doses. Our results highlighted that *n*-3 PUFA contents in a 50 g canned bluefin tuna portion would be almost equivalent to and potentially safer than those of 1 FOPF capsule (1000 mg)/die usually suggested for hyperlipidaemic subjects. Thus, Italian commercial canned bluefin tuna could be indicated as a functional food with potential health benefits for the prevention and care of cardiovascular disorders.

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1. Introduction

The European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) establishes that food is “functional” whether its positive effects on one or more organism functions are clearly proved so to be determinant for optimizing health conditions and/or decreasing disease incidence, independently from its nutritional capacity. Moreover, functional food should exert its potential bioactivities through quantities usually planned in a conventional dietary regimen (Diplock et al., 1999). Functional foods can be divided into “conventional” and “modified”. The first category consists in foods whose bioactive components are original constituents. The second category includes foods whose biological potential has been technologically influenced through: addition of bioactive compounds; removal of antinutrients or toxic substances; replacement of original components with exogenous bioactive ones; improvement of the bioavailability of original bioactive constituents.

The correlation between fish consumption and reduction of cardiovascular disease risk has focused scientific research attention since the seventies of the last century. The protective effects can be appreciated even when consumption levels are not elevated: a dietary regimen including at least 30 g fish/day can promote a significant prevention against cardiovascular disorders; particularly, an increase of 20 g/day in fish consumption would lead to a decrease by 7% of death risk for cardiovascular disease in subjects who occasionally consume fish (Mozaffarian et al., 2003). Although the biochemical mechanism of these beneficial properties have not been clearly explained so far, the positive effects of fish consumption would be mainly correlated to fish content in omega 3 long chain polyunsaturated fatty acids (*n*-3 PUFA), particularly, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), of which fish represents the main dietary source for man (Arino et al., 2005). The levels of these two fatty acids are strongly variable, both among the different fish species and within the same species, due to the type of diet and breeding. DHA and EPA are not directly produced by fish organism; they derive from unicellular algae occurring in the food chain (Arterburn et al., 2006). Assumption levels of DHA and EPA negatively correlate with the incidence of

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FOPF, fish oil based pharmaceutical formulation; *n*-3 PUFA, omega 3 long chain polyunsaturated fatty acids; ROS, Reactive Oxygen Species; SBTDEM, Simulated Bluefin Tuna DHA and EPA Mixture.

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degenerative diseases, such as cardiovascular, neurodegenerative and immune disorders. It has been reported that *n*-3 PUFA intake can reduce triglyceride plasma levels and platelet aggregation, and stabilizes the cardiac rhythm (Harris, 2009). Typical Western diet provides relatively small amounts of *n*-3 PUFA, probably inadequate to protect the body from chronic degenerative diseases. This deficit has been indicated as one of the main causes of the growing incidence of chronic diseases in our society. According to the American Heart Association Nutrition Committee, two servings of fish per week should be consumed for the prevention of cardiovascular diseases (Kris-Etherton et al., 2002). Previous studies have revealed that EPA and DHA would exert their best activity through a synergistic action and that a EPA/DHA ratio of 0.9:1.5 would be adequate for ideal cardiovascular protection (Mori and Woodman, 2006). In fact, this is precisely the respected ratio in most of pharmaceutical formulations based on fish oil *n*-3 PUFA extracts and indicated for the control of serum triglycerides in hyperlipidaemic subjects or for the secondary prevention in patients with previous myocardial infarction.

In the Western world, it is frequently overlooked that fish often means canned fish. Tuna, mackerel, sardines, salmon, and so on, reach our tables mainly as canned products, keeping intact most of their nutritional characteristics in quality controlled and long shelf-life packaging, which greatly facilitate, even in areas not close to the sea, the possibility of ideal fish consumption levels. Nevertheless, the biological potential of canned products can be significantly different respect to raw fish mainly due to thermal processing (fish steam-cooking and, then, can sterilization). Particularly, the deleterious effects of high temperatures on canned fish *n*-3 PUFA, mainly DHA and EPA, have been previously reported (Stephen et al., 2010; Miklavčič et al., 2011). Thus, the aim of the present work was to analyze the lipid profile of the most common canned fish species brands in the Italian market in order to evaluate their potential health benefits. The attention was focused on the products canned in brine considered as the closest to raw fish, particularly as regards their lipid profile, if compared to the ones canned in edible oils.

2. Materials and methods

2.1. Reagents and standards

All chemicals and reagents used were either analytical-reagent or GC grade. All organic solvents were purchased from Carlo Erba, Milano (Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA) before use. The standard mixture of fatty acid methyl esters, the pure standards *cis*-4,7,10,13,16,19-Docosahexaenoic acid (DHA, ⩾98%) and *cis*-5,8,11,14,17-Eicosapentaenoic acid (EPA, ⩾99%), were purchased from Sigma Chemicals (Milan, Italy).

2.2. Sample collection and lipid extract preparation

Fifty-one cans different fish species of various common Italian brands were obtained from retail trades. For each fish species, different numbers of brands were chosen as follows: seven, for *Thunnus thynnus* (bluefin tuna); six, for *Thunnus albacares* (yellowfin tuna); eight, for *Thunnus alalunga* (albacore); seven, for *Scomber scombrus* (Atlantic mackerel); five, for *Engraulis encrasicolus* (anchovy); six, for *Sardina pilchardus* (sardine); five, for *Xiphias gladius* (swordfish); seven, for *Salmo salar* (salmon). For each brand, six lots were taken into consideration. All of the samples had been canned in brine.

Aliquots (5 g) of each sample were freeze-dried and stored at −20 °C until analysis. Around 0.5 g of powder samples were subjected to lipid extraction according to AOAC method 948.16, by using a 6-place units Extraction Unit E-816 Soxhlet (Buchi, Flawil, Switzerland). After centrifugation at 3000*g* for 5 min, supernatants were transferred into a pre-weighed scintillation vial, and dried under nitrogen.

2.3. Analysis of fatty acid composition

Lipid extracts (0.1 g) were dissolved in 2 mL of *n*-eptane and treated with 0.2 mL of 2 N potassium hydroxide methanolic solution (11,2 g of potassium hydroxide in 100 mL methanol). The mixture was shaked energically for 1 min at room temperature and then centrifuged (3000*g* for 5 min). Supernatants were collected and analysis of fatty acid methyl esters was performed by gas chromatography using a DANI GC instrument (DANI Instruments, Milan, Italy) coupled to a flame ionization detector (FID) and equipped with a HP-5 capillary column (Agilent, Milan, Italy). The temperature programme started at 150 °C (10 min), increased by 2 °C/min to 180 °C and then increased again by 3 °C/min to 240 °C (20 min).

2.4. In vitro tests

2.4.1. Preparation of samples for in vitro tests

A mixture of commercial DHA and EPA standards was prepared simulating the same relative proportions as in canned bluefin tuna lipid profile, and has been referred to as SBTDEM (Simulated Bluefin Tuna DHA and EPA Mixture).

A typical commercial fish oil based pharmaceutical formulation (FOPF) indicated for the control of plasmatic lipid levels was chosen for the in vitro tests. It consisted in 1000 mg capsules containing PUFA ethyl esters with 85% minimum levels of EPA and DHA (ratio 0.9–1.5) and the following excipients: D,L α-tocopherol, gelatin succinate, glycerol, ethyl *p*-oxybenzoate, propyl *p*-oxybenzoate. Lipid extraction was performed as described in Section 2.2.

2.4.2. Cell culture and viability test

Rat cardiac H9C2 cells (ATCC, Manassas, VA) were cultured (17–21 passages) in DMEM supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin in 150 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2. The cells were fed every 2–3 days, and subcultured once they reached 70–80% confluence. Cell viability and proliferation were assessed by incubating the culture with SBTDEM and FOPF lipid extract samples (0.01–2 μg), and doxorubicin 1 μM for 72 h. Lipid extract samples were solubilized by adding 150 μL of DMSO and by mixing it in an orbital shaker for 5 min. As a control, 0.5% DMSO was added to untreated cells.

2.4.3. Preparation of cell extract

Cardiac H9C2 cells were collected by centrifugation and then resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 2 mM EDTA. The cells were sonicated, followed by centrifugation at 13,000*g* for 10 min at 4 °C. The resulting supernatants were collected and kept on ice for immediate measurements, as described below.

2.4.4. Measurement of intracellular ROS accumulation

2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA, 5 μM) was used to detect intracellular ROS levels in H9C2 cells (Vanden Hoek et al., 1997). DCF-DA is cell membrane permeable. Once inside the cells, DCF-DA is hydrolyzed by cellular esterases to form DCF, which is trapped intracellularly due to its membrane impermeability. DCF then reacts with intracellular ROS to form the fluorescent product, 2′,7′-dichloroflurosceín. Then, the cells were washed once with phosphate buffered saline (PBS) and lysed in 3 mL ice-cold 10 mM Tris–HCl buffer (pH 7.4) containing 0.2% sodium dodecyl sulfate. The cell lysates were collected and centrifuged at 2000*g* for 5 min at 4 °C. The fluorescence of the supernatants was measured using a Perkin–Elmer luminescence spectrometer (LS50B) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

2.4.5. Measurement of cellular superoxide dismutase activity

Total cellular superoxide dismutase (SOD) activity was measured as follows (Kirshenbaum and Singal, 1992). Briefly, a reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 1.33 mM diethylenetriaminepentaacetic acid, 1.0 U/mL catalase, 70 μM nitroblue tetrazolium, 0.2 mM xanthine, 50 μM bathocuproinedisulfonic acid, and 0.13 mg/mL bovine serum albumin (BSA). A 0.8 mL aliquot of the reaction mixture was added to each cuvette, followed by addition of 100 μL of lysate. The cuvettes were pre-warmed at 37 °C for 3 min. The formation of formazan blue was monitored at 560 nm, 37 °C for 5 min. The sample total SOD activity was calculated using a concurrently run SOD (Sigma) standard curve, and expressed as units per mg of cellular protein. Cellular protein content was quantified with Bio-Rad protein assay dye (Hercules, CA) based on the method which makes use of BSA as the standard.

2.4.6. Measurement of caspase-3 activity

Caspase-3 activity was measured using the BD ApoAlert Caspase-3 Fluorogenic Assay (BD Biosciences Clontech, Palo Alto, CA). Briefly, protein lysates were collected from cells that had been incubated with lioRGJ (0.01–1 μg) for 8 h, as per protocol. Activity was measured using a fluorescent microplate reader (PerSeptive Biosystems, Farmington, MA).

2.5. Statistics

Unless otherwise stated, all of the experimental results were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was performed by the Student's *t* test or two-way ANOVA followed by

Table 1
Lipid profile of different Italian commercial canned fish species.*

Species	FAO name	Total lipid	SAFA	MUFA	PUFA	n-3 PUFA	EPA	DHA	n-6 PUFA
Thunnus thynnus	Bluefin tuna	5.64 ± 0.44^{a}	$1.55 \pm 0.24^{a,b}$	$1.51 \pm 0.37^{a,e}$	2.57 ± 0.10^{a}	2.32 ± 0.17^{a}	0.79 ± 0.10^{a}	1.52 ± 0.25^{a}	0.25 ± 0.04^{a}
Thunnus albacares	Yellowfin tuna	3.74 ± 0.52^{b}	$1.41 \pm 0.12^{a,b}$	0.70 ± 0.11^{b}	1.61 ± 0.32^{b}	$1.49 \pm 0.39^{b,d}$	$0.14 \pm 0.03^{b,e}$	$1.28 \pm 0.22^{a,b,e}$	0.11 ± 0.02^{b}
Thunnus alalunga	Albacore	$4.70 \pm 0.31^{c,e}$	$1.75 \pm 0.22^{a,b}$	$1.51 \pm 0.29^{a,e}$	1.43 ± 0.28^{b}	$1.28 \pm 0.22^{b,d}$	$0.22 \pm 0.04^{b,c}$	$1.04 \pm 0.13^{b,c,e}$	0.14 ± 0.03^{b}
Scomber scombrus	Atlantic mackerel	5.73 ± 0.47^{a}	$1.92 \pm 0.41^{a,b,c}$	$2.31 \pm 0.40^{c,f}$	1.48 ± 0.19^{b}	$1.10 \pm 0.13^{b,c}$	0.24 ± 0.04^{c}	$0.85 \pm 0.10^{c,d}$	0.37 ± 0.07^{c}
Engraulis encrasicolus	Anchovy	$4.04 \pm 0.20^{b,d}$	1.28 ± 0.34^{b}	$1.18 \pm 0.14^{a,b,c}$	1.54 ± 0.16^{b}	$1.46 \pm 0.31^{b,d}$	0.54 ± 0.08^{d}	$0.91 \pm 0.10^{c,d,e}$	0.074 ± 0.006^{b}
Sardina pilchardus	Sardine	$4.63 \pm 0.19^{d,e}$	$1.64 \pm 0.18^{a,b}$	$1.27 \pm 0.33^{a,b}$	1.71 ± 0.13^{b}	$1.44 \pm 0.11^{b,d}$	0.51 ± 0.07^{d}	$0.92 \pm 0.10^{c,d,e}$	0.26 ± 0.04^{a}
Xiphias gladius	Swordfish	4.58 ± 0.36^{e}	$1.60 \pm 0.13^{a,b}$	2.97 ± 0.26^{d}	0.85 ± 0.21^{c}	0.77 ± 0.25^{c}	0.11 ± 0.02^{e}	0.65 ± 0.14^{d}	0.071 ± 0.004^{b}
Salmo salar	Salmon	6.46 ± 0.71^{f}	1.96 ± 0.23^{c}	$2.08 \pm 0.73^{e,f}$	2.41 ± 0.39^{a}	1.58 ± 0.26^{d}	0.38 ± 0.06^{f}	1.19 ± 0.22^{e}	0.82 ± 0.11^{d}

[a,b,c,d,e,f]Mean values in columns with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test ($P < 0.05$).
SAFA: total saturated fatty acids; MUFA: total monounsaturated fatty acids; PUFA: total polyunsaturated fatty acids; EPA: eicosapentaenoic acid (20:5*n*-3); DHA: docosahexaenoic acid (22:6*n*-3).
* Data are expressed as mean value (g/100 g fresh weight) ± SD ($n = 5$).

the Tukey–Kramer multiple comparison test to evaluate significant differences between a pair of means. The level of significance (α-value) was 95% in all cases ($P < 0.05$).

3. Results and discussion

The quali-quantitative lipid pattern of the commercial canned fish samples analyzed is reported in Table 1. Bluefin tuna meat revealed a higher percentage of *n*-3 PUFA in comparison with other commercial fish species. Particularly, *n*-3 PUFA in bluefin tuna accounted for 41.1% of total lipid respect to a range of 16.8–39.8% of the other commercial samples, and DHA represented an important constituent (26.9% *vs* a range of 14.2–34.2% of total lipid) with the exception of yellowfin tuna (34.2%). As regards the absolute values (g/100 g), bluefin tuna demonstrated the highest amount of *n*-3 PUFA and DHA, while salmon was the highest for SAFA and *n*-6 PUFA (Table 1). A *n*-3/*n*-6 PUFA ratio of 1:6 is considered to be adequate for the nutritional needs for most healthy adults, particularly when *n*-3 PUFA consist mainly of EPA and DHA. Such ratio would exert an important influence on plasma lipids and serve cardiac and endothelial functions to impact the prevention and treatment of coronary heart diseases (CHD) (Wijendran and Hayes, 2004; Makni et al., 2010). Today, this balance in the usual Western diet has been severely shifted, and the amount of *n*-3 PUFA in the diet has decreased, while the amount of *n*-6 PUFA has increased. A dietary intake of fish with high ratio of *n*-3/*n*-6 PUFA would therefore be beneficial (Wijendran and Hayes, 2004). Bluefin tuna revealed not only an interesting *n*-3/*n*-6 PUFA ratio (9.3 *vs* a range of 1.9–19.7) but, in particular, the highest amount of *n*-3 PUFA among the commercial samples analyzed (Table 1).

Clinical trials and experimental studies suggest that EPA and DHA have important antiatherogenic and antithrombotic properties deriving from a wide range of biological effects, including benefits on lipoprotein metabolism, blood pressure, endothelial function and vascular reactivity, inflammation, platelet and fibrinolytic function, cytokine production, coagulation and oxidative stress (Mori and Woodman, 2006). The favorable effects of fish oils were primarily attributed to EPA, despite the fact that some fishes provide substantial quantities of DHA. Even until recently, it was unclear as to whether EPA or DHA were equally important in relation to cardiovascular protection. A limiting factor has been the lack of sufficient quantities of purified EPA or DHA, resulting in the individual effects of EPA and DHA in humans being examined in only a few controlled trials. These data now demonstrate that DHA and EPA have both important haemodynamic and anti-atherogenic properties, although they show independent effects on cardiovascular risk factors in humans. Our experimental results revealed that the EPA/DHA ratio in bluefin tuna sample was the closest to the ideal one occurring in most of fish oil based pharmaceutical formulations (FOPF) among the commercial samples tested. Our data regarding the EPA and DHA contents in the commercial fish samples analyzed have been compared with the one of a typical FOPF of which 1 capsule (1000 mg)/day is usually suggested for the control of serum triglycerides in hyperlipidaemic subjects or for the secondary prevention in patients with previous myocardial infarction. Interestingly, our results revealed that the EPA and DHA contents in a 50 g bluefin tuna portion are almost equivalent to those of 1 capsule (1000 mg) of the same FOPF (Table 2).

The correlation between intake of fish oil *n*-3 PUFA, mainly DHA and EPA, and reduction of cardiovascular disease risk (Arino et al., 2005), may indicate cardiac cell lines as a suitable model system for testing the potential effects of fish oil extracts and their derivatives on the cardiovascular physiology. A mixture of commercial DHA and EPA standards was prepared simulating the same relative proportions as in canned bluefin tuna lipid profile, and has been referred to as SBTDEM (Simulated Bluefin Tuna DHA and EPA Mixture). The effect on cardiac derived H9C2 myocytes proliferation exposed to increasing doses (0.01–1.8 μg) of SBTDEM and FOPF lipid extracts was examined (Fig. 1). SBTDEM revealed a linear correlation between incubation dose and cell proliferation, and the maximum result was achieved with a 0.1 μg dose which made cell proliferation increase by about 95%. The positive influence of *n*-3 PUFA cell membrane incorporation on the control of cell functions relevant to cardiovascular diseases has already been reported. Assimilation of *n*-3 PUFA into plasma membrane phospholipids has major effects on cell signaling by altering membrane fluidity, lipid raft structure, and substrate availability for the synthesis of bioactive oxidized fatty acids (Jump, 2002). DHA plays a key structural role in membrane architecture; this highly unsaturated fatty acid alters membrane fluidity, membrane cholesterol content, and lipid raft organization (Wassall and Stillwell, 2009; Soni et al., 2008). The impact on intracellular calcium handling is also particularly noteworthy. Stanley et al. (2012) recently identified the mitochondrial permeability transition pore (MPTP) as a target for *n*-3 PUFA regulation. MPTP is a large-diameter, high-conductance,

Table 2
Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) contents of commercial canned bluefin tuna *vs* a typical fish oil based pharmaceutical formulation (FOPF).*

	Bluefin tuna (g/50 g)	FOPF capsule 1000 mg (g × cps)
DHA	0.76 ± 0.55	0.62 ± 0.83
EPA	0.39 ± 0.40	0.38 ± 0.37

* Data are expressed as mean value (g/100 g fresh weight) ± SD ($n = 5$; $P < 0.05$).

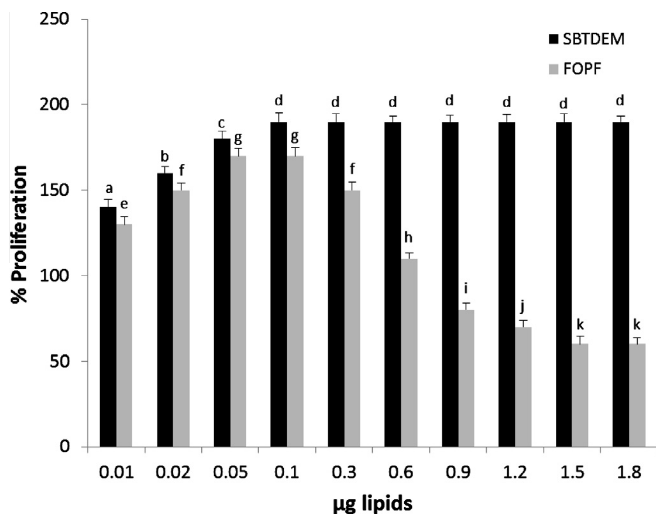

Fig. 1. Effect of a Simulated Bluefin Tuna DHA and EPA Mixture (SBTDEM) and a typical fish oil based pharmaceutical formulation (FOPF) on H9C2 cardiomyocyte proliferation. The 100% proliferation refers to untreated cells. Values are expressed as means ± SD ($n = 5$; $P < 0.05$). [abcdefghijk]Mean values with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test.

voltage-dependent channel that allows passage of water, ions, and molecules up to 1.5 kDa. Mitochondrial accumulation of Ca^{2+} induces MPTP opening, whereas Ca^{2+} chelation closes the channel. Opening of MPTP is linked to cell death and has been implicated in ischemic injury and heart failure (Stanley et al., 2012; Duda et al., 2007). The EPA-derived epoxide, 17(R),18(S)-epoxyeicosatetraenoic acid, has anti-arrhythmic effects; it rapidly and with high potency ($EC_{50} \sim 1–2$ nM) protects neonatal cardiomyocytes against Ca^{2+} overload (Falck et al., 2011). An additional mechanism at the base of the potential cardiovascular protection is the nuclear effect of *n*-3 PUFA on the inflammation process. NFκB is a major transcription factor regulating expression of genes encoding proteins involved in inflammation; some target genes include COX2, cytokines (e.g., TNFα), and chemokines (e.g., MCP1) (Ben-Neriah and Karin, 2011). *n*-3 PUFA, particularly, elevated cell membrane DHA levels, have been reported to suppress the nuclear levels of NFκB in several model systems and down-regulate acid sphingomyelinase activity, leading to a decline in ceramide, an inflammatory and pro-apoptotic lipid (Jump, 2004).

Similarly, data reported in Fig. 1 demonstrated that FOPF lipid extract at a maximum sample dose of 0.1 µg was able to exert positive effects on cardiac cell proliferation. Nevertheless, exposure to increasing concentrations resulted in a reversal of the observed trend, and at a sample dose of 0.9 µg cytotoxic effects were revealed. These results are consistent with what has been stated in literature for fatty acid ethyl esters of which most of FOPF are based. Fatty acid ethyl esters would be 50 times more resistant to pancreatic lipase as compared to hydrolysis of triglycerides, so that in part they would be bioavailable in their integral form (Yang et al., 1990a,b). Once specific target tissues are reached, fatty acid incorporation in the cell phospholipid bilayer would imply trans-esterification and consequent cell damages due to the amount of ethanol released (Best and Laposata, 2003). Moreover, it has been demonstrated that *n*-3 PUFA in the form of ethyl esters are much less stable than those in the natural triglyceride form and readily oxidize producing cytotoxic radical products (Yoshii et al., 2002).

In order to ascertain the potential effects of canned bluefin tuna *n*-3 PUFA on the doxorubicin-induced oxidative stress in cardiac cells, H9C2 cardiomyocytes were exposed to 1 µM doxorubicin and a combination of doxorubicin and different doses of SBTDEM and FOPF lipid extracts for 72 h (Table 3). Maximum aliquot of 0.1 µg SBTDEM provided an appreciable radical-scavenging activity as indicated by the decrease in the free-radical levels (especially ROS species, about 31%) and the unchanged antioxidant defense system activity (Table 3). Similarly, the association of doxorubicin with maximum FOPF lipid extract dose of 0.1 µg revealed positive effects on induced cardiac cell oxidative stress. These results would confirm that dietary *n*-3 PUFA supplementation is associated with a reduction in the susceptibility of myocytes to ROS-induced injury and this may be related to membrane incorporation of *n*-3 PUFA, increased antioxidant defense systems, changes in cardiomyocyte membrane fluidity, and the ability to prevent rises in cellular Ca^{2+} in response to ROS (Jahangiri et al., 2006). On the contrary, the co-administration of higher FOPF lipid extract doses led to the enhancement of cardiac cell oxidative stress, probably due to the sample pro-oxidant effects (ethanol released and secondary unstable oxidized derivatives), as indicated mainly by the increase in RNS and antioxidant enzyme levels (Table 3).

To confirm such hypothesis, the influence of 1 µM doxorubicin and a combination of doxorubicin with different SBTDEM and FOPF lipid extract doses on caspase-3 activity in cardiomyocytes was assayed (Fig. 2). Among the many known regulators and effectors of apoptosis, caspases are a family of cytoplasmic proteases that plays an important role in the execution phase of apoptosis. Two groups of caspases can be identified: upstream initiator caspases, that cleave and activate other caspases, and downstream effector caspases, including caspase-3, caspase-6, and caspase-7, that cleave a variety of cellular substrates or inactivating enzymes. Caspase-3 is a central executioner in apoptosis (Thornberry and Lazebnik, 1998). Cells were incubated with doxorubicin and a combination of doxorubicin and different doses of SBTDEM and FOPF lipid extracts in medium for 8 h and then lysed to measure caspase-3 activity using a fluorogenic assay. Our results showed that doxorubicin significantly up-regulated caspase-3 activity while its combination with maximum sample aliquot of 0.1 µg SBTDEM seemed to effectively depress (by about 50%) the activity of this apoptotic factor (Fig. 2). An almost comparable result was obtained with 0.1 µg of FOPF lipid extract. Interestingly, an increase in FOPF lipid extract dose exposure to cardiomyocytes seemed to be less effective in reducing caspase-3 activity. Collectively, these data suggested that higher doses of FOPF lipid sample caused cell death via the caspase-3-mediated apoptotic pathway.

Table 3
Effect of a simulated bluefin tuna DHA and EPA mixture (SBTDEM) and a typical fish oil based pharmaceutical formulation (FOPF) on doxorubicin-induced oxidative stress in lysate of H9C2 cardiomyocytes.*

	Control	Dox 1 µM	Dox 1 µM + 0.1 µg SBTDEM	Dox 1 µM + 0.1 µg FOPF	Dox 1 µM + 0.9 µg FOPF
TBARS µM/µg protein	0.0043 ± 0.0005[a]	0.0068 ± 0.0008[b]	0.0021 ± 0.0003[c]	0.0034 ± 0.0003[d]	0.0088 ± 0.0007[e]
NO_2^- nmol/µg protein	0.0010 ± 0.0004[a]	0.0065 ± 0.0007[b]	0.0022 ± 0.0003[c]	0.0032 ± 0.0004[d]	0.0240 ± 0.001[e]
MnSOD U/µg protein	0.0100 ± 0.001[a]	0.0180 ± 0.002[b]	0.0080 ± 0.0006[c]	0.0110 ± 0.001[a]	0.0350 ± 0.001[d]

[a,b,c,d,e]Mean values in rows with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test.
Dox: doxorubicin; TBARS: Thiobarbituric Acid Reactive Substances; MnSOD: manganese superoxide dismutase; Control: untreated cell lines.
* Values are expressed as means ± SD ($n = 5$; $P < 0.05$ compared to the control).

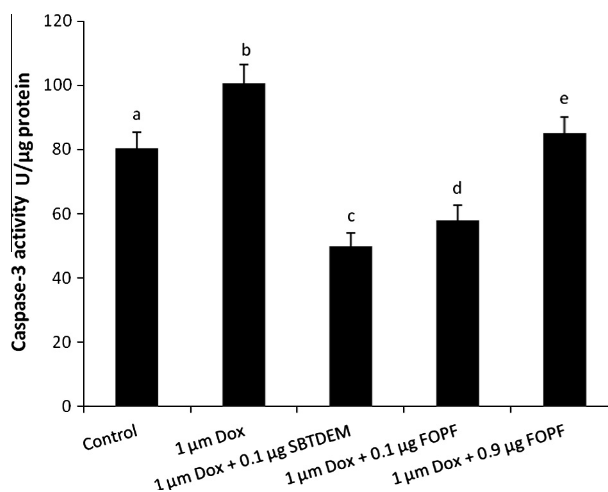

Fig. 2. Effect of doxorubicin (Dox) in association with a Simulated Bluefin Tuna DHA and EPA Mixture (SBTDEM) and a typical fish oil based pharmaceutical formulation (FOPF) on caspase-3 activity in lysate of H9C2 cardiomyocytes. Values are expressed as means ± SD ($n = 5$; $P < 0.05$). [abcde]Mean values with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test. Control: untreated cell lines.

4. Conclusion

Canned bluefin tuna showed the most interesting lipid profile, as regards their potential health benefits, among the different Italian commercial canned fish species analyzed. Tests on H9C2 cardiomyocytes revealed that bluefin tuna *n*-3 PUFA may be responsible for a significant cell protection against both physiological and doxorubicin-induced oxidative stress. Analogous tests performed by incubating cardiac cells with FOPF lipid extract showed cytotoxicity at high doses confirming that fatty acid ethyl esters, of which most of FOPF are based, would release ethanol and would be less stable to oxidative processes than triglycerides. Our results highlighted that EPA and DHA contents in a 50 g canned bluefin tuna portion would be almost equivalent to and potentially safer than those of 1 FOPF capsule (1000 mg)/day usually suggested for the control of serum triglycerides in hyperlipidaemic subjects or for the secondary prevention in patients with previous myocardial infarction. Thus, canned bluefin tuna could be indicated as a functional food potentially useful for the prevention and care of cardiovascular disorders. Undoubtedly, further *in vivo* and clinical studies are needed to support these results.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2014.06.016.

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