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Protective Effects of *Plathymenia reticulata* and *Connarus favosus* Aqueous Extracts against Cadmium- and Mercury-Induced Toxicities

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Abstract

The extracts of *Plathymenia reticulata* and *Connarus favosus* are widely used in the folk medicine. The potential protective effects of these extracts have been evaluated against cadmium in the yeast *Saccharomyces cerevisiae*, and against mercurial contamination in zebrafish *Danio rerio*. In yeast, both extracts efficiently protected the Δ*ycf1* mutant strain exposed to cadmium chloride restoring the growth, the expression of stress-response genes and decreasing the level of oxidative stress. In zebrafish, the supplementation of methylmercury-contaminated diet with both plant extracts similarly protected fish through the suppression of the methylmercury-induced lipid peroxidation, decrease of acetylcholinesterase activity, and restoring the expression levels of stress-response genes. This study particularly demonstrates the protective potential of both aqueous extracts against methylmercury, and could represent an interesting alternative for the Amazonian fish-eating communities to cope with the impact of chronic exposure to contaminated diets.

***Key words*:** Plant extract, Methylmercury, Cadmium, *Saccharomyces cerevisiae*, *Danio rerio*, Oxidative stress

INTRODUCTION

Since the dawn of civilization, medicinal plants have been used to alleviate diseases and afflictions. Furthermore, they are still currently widely used as the sole therapeutic option for many isolated communities in Brazil, where ethnobotanical surveys have confirmed the presence of several species endowed with medicinal properties (1-4).

Recently, two aqueous extracts were chemically characterized. The first, from *Plathymenia reticulata* Bentham (Fabaceae), is rich in condensed tannins and phenolic compounds (5), while the second, from *Connarus favosus* Planch. (Connaraceae) is rich in condensed tannins, phenolic compounds, flavonoids, and catechins (6). These extracts are widely used in folk medicine, particularly to treat snakebites (7). Their efficacy as snakebite anti-venoms has been confirmed *in vitro* through the inhibition of the hemorrhagic activity of *Bothrops atrox* venom (5,6). However, several other medicinal properties need to be validated. *P. reticulata* is known by the vernacular name candeia or vinhático (8) and is used to treat inflammation, pain, and kidney troubles (9).

Humans, especially Amerindian and riverine populations in the Amazonian basin are exposed to hazardous

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mercurial contamination mainly through fish consumption, and the consequences are decreased motor performance and visual impairment (10). The use of elemental liquid mercury (Hg^0) by clandestine gold miners and the natural richness of the Amazonian soils in inorganic Hg has led to the production of methylmercury (MeHg) in the aquatic environment through the activity of anaerobic bacteria. MeHg biomagnifies up the food chain and carnivorous fish eventually become highly contaminated (10). A diet rich in fruits would decrease the mercury uptake through fish consumption in human communities in the Brazilian Amazon (11,12). Therefore, it is fairly possible that the use of traditional plant extracts may play a protective role against mercurial exposure.

The goal of this study was to evaluate the potential protective effects of both extracts against metal-induced toxicity, following direct exposure to cadmium chloride ($CdCl_2$) in yeast. In addition, we also investigated the protective effects against chronic contamination of zebrafish with mercury-contaminated food mimicking the level of exposure of carnivorous fish in the Amazonian basin (10). At the end of the 60-day exposure period, the mercury accumulation levels of the brains, livers, and skeletal muscles of the fish were measured as well as the lipid peroxidation, acetylcholinesterase (AChE) activity, and expression of a selected subset of genes. Indeed, MeHg is known to inhibit AChE activity in mammalian species and fish, disrupting the mitochondrial respiratory chain, thereby increasing the reactive oxygen species levels, altering calcium homeostasis, and increasing lipid peroxidation (13-18).

MATERIALS AND METHODS

Aqueous plant extract preparation. Two tree species of Amazonia have been used to extract useful tannins: the bark of *P. reticulata* Bentham (Fabaceae), and the inner bark of *C. favosus* Planch. (Connaraceae). Samples of the inner bark of *C. favosus* were collected in São Pedro community, Santarém, Pará, Brazil (2° 32′ 8.9″ S and 54° 54′ 23.9″ W). Samples of *P. reticulata* bark were collected close to the community of Cucurunã, Santarém, Pará, Brazil (2° 26′ 53.9″ S and 54° 47′ 5″ W). Water extraction of these vegetal materials and determination of their major chemical class of compounds was performed as previously described for the aqueous extracts of *P. reticulata* (5) and *C. favosus* (6).

Yeast culture and selection of working extract doses in zebrafish feed. The potential protective effects of *P. reticulata* and *C. favosus* extracts were first investigated in the Δ*ycf1* mutant yeast *Saccharomyces cerevisiae* challenged with toxic doses of $CdCl_2$ (20 or 40 μM, known to trigger the onset of an oxidative stress and DNA damage) using two concentrations of both extracts (500 or 1,000 mg/L) and monitoring the biomass growth. This mutant strain is hypersensitive to drugs and cadmium because it lacks a vacuolar ATP-binding cassette transporter, which allows vacuolar transfer and accumulation of glutathione (GSH)-conjugated drugs and cadmium (19). Additionally, changes in the expression patterns of genes related to the oxidative and mutagenic stress response (*GSH1*, *KAR2*, paired related homeobox 1 (*PRX1*), and RNA, ribosomal 45S cluster 3 [*RNR3*]) were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). These genes were selected because they are well known to be upregulated when the oxidative and mutagenic status of the cell is increased (20,21). This allowed the selection of an effective dose of the extract to be incorporated in the zebrafish feed. The Δ*ycf1 S. cerevisiae* strain was cultivated as described in the Additional file. The biomass growth was assessed by quantifying the number of copies of the *GSH1* gene as described in the Additional file. The qRT-PCR analysis of the yeast mentioned above gene expression was carried out as described in the Additional file (Supplementary Table 1).

Zebrafish culture and experimental diet preparation. The experimental diets for the zebrafish (supplemented, contaminated, or both) were prepared by mixing artificial fish food (Dr. Bassleer Biofish, Telgte, The Netherlands) with reagent-grade alcohol (95% ethanol, Prolabo, France) and 0.5% (5 mg extract per g of food) of the plant aqueous extract or dissolved methylmercury chloride (reference 33368, Sigma-Aldrich), or both as previously described (22). Wild-type adult zebrafish were purchased from a commercial company (Exomarc, Lormont, France), and acclimatized for 7 days at 23°C in a large tank filled with dechlorinated water (body weight: 0.79 ± 0.03 g, wet weight; standard length: 3.33 ± 0.07 cm, $n = 6$). Twenty adult fish were randomly placed in six different plastic tanks with 20 L of dechlorinated water, continuously aerated by bubbling air, and were daily given a quantity of food representing 5% of their body weight (b.w.). Control fish were fed with uncontaminated food (Dr. Bassleer Biofish, Telgte). Under the exposure conditions designated "MeHg," the fish were fed contaminated food containing 13.5 μg MeHg/g dry weight (d.w.). Under the assumption that 100% of the ingested MeHg is absorbed, the zebrafish were exposed to 675 μg $Hg \cdot day^{-1} \cdot kg\ b.w^{-1}$. This concentration of MeHg was chosen because of its environmental relevance to the mercury content of fish eaten by other piscivorous fish and based on our previous studies showing that it decreased mitochondrial respiration and ATP synthesis rates in skeletal muscles (23). In addition, it disorganized the mitochondrial cristae structure of the skeletal muscles (24), induced a strong MeHg demethylation process in the liver (22), and impaired the optical tectum integrity, with decreased cell density (25). In the

exposure condition hereafter called "*P. reticulata*" or "*C. favosus*," the fish were fed with control food supplemented with 0.5% (w/w) of either the *P. reticulata* or *C. favosus* extract at an average dose of ingested extract equal to 250 $mg \cdot kg^{-1} \cdot day\ b.w.^{-1}$. This concentration was chosen based on the results observed in yeast exposed to $CdCl_2$ and treated with the extracts. Indeed, at a concentration of 500 mg/L, both extracts exerted a potent beneficial effect on yeast by increasing the survival and decreasing oxidative stress after a $CdCl_2$ challenge (see the following results). Similarly, under the exposure conditions hereafter designated "*P. reticulata* + MeHg" and "*C. favosus* + MeHg", the food was spiked with 13.5 µg MeHg/g d.w. and supplemented with 0.5% of either the *P. reticulata* or *C. favosus* extract. To minimize fish contamination by the water, half of the water volume from each tank was changed every 2 days, and tank bottoms were cleaned every day to eliminate fish feces, and food remains.

After 60 days of exposure to the different diets, the fish were collected, euthanized within seconds by immersion in melting ice in agreement with the ethical guidelines displayed and used by the NIH intramural research program. Then, the fish were dissected to assess the mercury accumulation, mercurial neurotoxicity, lipid peroxidation, and gene expression.

Mercury quantification. The mercury concentrations in the food (13.6 ± 0.90 µg Hg/g d.w., $n = 3$) and fish tissues were determined using flameless atomic absorption spectrometry, and carried out automatically after thermal decomposition at 750°C under an oxygen flow (AMA 254, Altec, Prague, Czech Republic) with a detection limit of 0.01 ng Hg. The precision (% of relative standard deviation) was 10%, and the accuracy of the analytical methodology was determined for each set of measurements by analyzing the certified reference material (CRM) in lobster hepatopancreas, *TORT-2*. CRM determined values: 0.27 ± 0.03 ng Hg/mg dry weight (mean ± SD, $n = 120$ measurements); CRM recommended value: 0.27 ± 0.06 ng Hg/mg d.w.).

Lipid peroxidation assessment. The level of lipid peroxidation has been investigated using the thiobarbituric acid reactive substances (TBARS) assay, which allows the quantification of oxidative stress by measuring the peroxidative damage to lipids that occurs with free radical generation. These damages to lipids are associated with the production of malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) under specific temperature and acidic condition, generating a chromogen measurable spectrofluorometrically. The TBARS assay was conducted according to a previously published protocol (26) using the 1,1,3,3-tetramethoxypropane (TMP, ref: 108383, Sigma-Aldrich, St Louis, MO, USA) as an external standard. The total protein content was measured using the Bradford method based on the ability of Coomassie blue to bind proteins. The levels of lipid peroxidation are expressed as nanomoles (nmol) of TBARS/mg of proteins.

Measurement of AChE activity. The activity of AChE, a neurotoxicity biomarker, was assessed in the brains and skeletal muscles of fish using the most common method (27) using microplate spectrometry. This assay is based on acetylthiocholine iodide cleavage by AChE and the subsequent reaction of thiocholine with the chromogenic disulfide 5,5-dithiobis 2-nitrobenzoic acid. The reaction was run for 5 minutes following the addition of the substrate, and the optical density (OD) was measured every 13 seconds. Then, the AChE activity was calculated using the molar extinction coefficient of the 5-thio-2-nitrobenzoic acid ($13{,}600\ L \cdot mol^{-1} \cdot cm^{-1}$) and expressed as nanomoles (nmol) per hydrolyzed substrate milligram protein per minute (mg protein/min). The total protein content was measured using the Bradford method based on the binding of Coomassie blue to proteins. We used the Bio-Rad protein assay dye reagent concentrate (ref: 5000006), and bovine serum albumin (BSA, ref: A2153, Sigma-Aldrich) as the standards.

Total RNAs extraction and qRT-PCR analysis. Total RNA was extracted from five whole brains, livers, and skeletal muscles using the Absolute RNA RT-PCR Miniprep kit (Stratagene, Agilent Technologies, Les Ulis, France) according to the manufacturer's protocol. To eliminate the maximum lipids and proteins, a phenol-chloroform-isoamylic alcohol (25 : 24 : 1) extraction step was added just after the homogenization step. Following the extractions, first-strand cDNA was synthesized from total the RNA using the Stratascript First-Strand Synthesis System (Stratagene, Agilent) according to the manufacturer's instructions. The cDNA samples were kept at −20°C until used in the real-time PCR reactions, which were performed using a thermocycler (MX3000P qPCR System, Stratagene, Agilent) using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's recommendations. The parameters set to perform the qRT-PCR reactions are provided in the Additional file. The primers used in the qRT-PCR reactions are given in Supplementary Table 2.

Statistical analyses. All statistical analyses were performed using three to five biological replicates per condition. First, the normality and homogeneity of the variance were tested using the Shapiro-Wilks test and Levene test respectively ($\alpha = 0.05$). If normality and homoscedasticity were confirmed, a Student's *t*-test was performed ($\alpha = 0.05$). Otherwise, the Student's *t*-test was replaced by the Mann-Whitney test ($\alpha = 0.05$).

Table 1. Effect of natural extracts on the growth of the yeast Δ*ycf1* mutant exposed to cadmium (in % of the cadmium-untreated and extract-free control yeast growth)

$CdCl_2$ (μM)	Without extract	*P. reticulata*		*C. favosus*	
		500 mg/L	1000 mg/L	500 mg/L	1000 mg/L
0	100 ± 6[a]	99 ± 2	99 ± 4	108 ± 8	100 ± 7
20	46 ± 3	81 ± 7[§]	91 ± 5[§]	92 ± 4[§]	122 ± 19[§]
40	36 ± 7	52 ± 5[§]	55 ± 5[§]	47 ± 5	48 ± 24

The yeast biomass has been assessed through the quantification of the number of copies of the *GSH1* gene by qPCR. Values are means ± SD ($n = 3$).

[a]The number of *GSH1* gene copies for the control without extract was equal to $(1.8 \pm 0.1) \times 10^5$ in the assay, corresponding to a Ct equal to 21.81 ± 0.08 ($n = 3$).

[§]This symbol indicates significant differences between growth values in the presence of natural extract compared to the extract-free control, for the same concentration of cadmium, as assessed by the Mann-Whitney test ($p < 0.05$).

RESULTS

Protection conferred by P. reticulata and C. favosus extracts to yeast against the effects of cadmium. To evaluate the protective properties of the natural extracts in the yeast mutant strain Δ*ycf1* exposed to $CdCl_2$, we monitored the growth (Table 1) and measured the expression levels of oxidative and mutagenic stress-response genes (Table 2).

Both natural extracts alleviated the cadmium-induced decreased growth (Table 1). Indeed, after exposure to 20 μM $CdCl_2$, which resulted in a 54% inhibition of growth, treatments with 500 and 1,000 mg/L of the *P. reticulata* extract significantly induced a growth increase of 35 and 45% compared to the cadmium-exposed condition, respectively (Table 1). Similar effects were observed after exposure to 40 μM $CdCl_2$ and treatment with 500 and 1,000 mg/L *P. reticulata* extract, which significantly increased the growth by 16 and 19%, respectively compared to the cadmium-exposed condition (Table 1). Treatment with the *C. favosus* extract also reduced the impact of $CdCl_2$ exposure on growth only at a concentration of 20 μM $CdCl_2$. Treatments with 500 and 1,000 mg/L *C. favosus* extract significantly increased the growth by 46 and 76%, respectively compared with the 20 μM cadmium-exposed condition (Table 1).

Additionally, we evaluated the protective effects of both natural extracts on the level of expression of four oxidative and mutagenic stress-response genes (Table 2). The exposure of the yeast mutant Δ*ycf1* to both $CdCl_2$ concentrations significantly and affected the expression levels of the studied genes, in a concentration-dependent manner (Table 2). Indeed, exposure to 20 μM $CdCl_2$ induced a 3- and 2-fold increase in the expression level of the *KAR2* and the *PRX1* genes compared to the control condition, respectively (Table 2). However, exposure to the highest concentration of $CdCl_2$ induced a significant over-expression of the four studied genes compared to the control levels, with 2.6-, 3.4-, 2.8-, and 5.5-fold increased levels of expression of *GSH1*, *KAR2*, *PRX1*, and *RNR3* genes, respectively (Table 2), indicating the expected onset of an

Table 2. Effect of natural extracts on the expression of yeast genes involved in the resistance to oxidative stress in the Δ*ycf1* mutant exposed to cadmium

$CdCl_2$ (μM)	Genes			
	GSH1	KAR2	PRX1	RNR3
0	25 ± 15	60 ± 34	46 ± 28	10 ± 8
20	39 ± 5	182 ± 28*	95 ± 3*	30 ± 6
40	66 ± 12*	204 ± 35*	130 ± 15*	55 ± 21*
	Plathymenia reticulata bark extract 500 mg/L			
0	7.5 ± 1.5	11 ± 6	3 ± 2	0.6 ± 0.3
20	2.3 ± 0.7[§]	6 ± 2[§]	3.8 ± 1.5[§]	0.45 ± 0.3[§]
40	2.7 ± 1.7[§]	15 ± 3[§]	2.2 ± 0.8[§]	0.3 ± 0.1[§]
	1000 mg/L			
0	1.5 ± 0.1	1.5 ± 0.7	2.2 ± 0.8	1.5 ± 0.8
20	4.5 ± 3.5[§]	2.2 ± 0.7[§]	3.8 ± 2.2[§]	0.5 ± 0.1[§]
40	1.2 ± 0.2[§]	5.0 ± 0.1[§]	4.5 ± 2.2[§]	1.0 ± 0.6[§]
	Connarus favosus inner bark extract 500 mg/L			
0	6 ± 2	7 ± 2	5 ± 2	8 ± 2
20	21 ± 7	33 ± 8[§]	22 ± 6[§]	22 ± 9
40	17 ± 6[§]	21 ± 9[§]	15 ± 7[§]	11 ± 6
	1000 mg/L			
0	14 ± 13	8 ± 3	12 ± 8	5 ± 0,4
20	7 ± 1[§]	14 ± 5[§]	8 ± 2[§]	8 ± 1[§]
40	0.5 ± 0.3[§]	1.7 ± 0.9[§]	0.6 ± 0.3[§]	0.7 ± 0.2*[§]

The gene of the 18S ribosomal RNA were used as reference. All values were multiplied by 1000 to facilitate reading. Values are means ± SEM ($n = 3$).

*This symbol indicates significant differences between the expression values in the presence of cadmium compared to cadmium-untreated control, for the same concentration of natural extract as assessed by the Mann-Whitney test ($p < 0.05$).

[§]This symbol indicates significant differences between the expression values in the presence of natural extract compared to the extract-free control, for the same concentration of cadmium, as assessed by the Mann-Whitney test ($p < 0.05$).

oxidative stress and damage to DNA.

The treatment of cadmium-exposed yeast mutant Δ*ycf1* with both natural extracts suppressed the cadmium-induced over-expression of the oxidant and mutagenic stress-response genes, and the protective effects were much prominent with the *P. reticulata* treatment that with *C. favosus*. Additionally, whatever the exposure concentration of $CdCl_2$, both concentrations of each extract maintained the expression level of these genes similar to those of the cadmium-untreated control, except for *C. favosus* extract at a concentration of 1,000 mg/L during a challenge with 40 μM of $CdCl_2$ (Table 2).

Hg accumulation in zebrafish organs. After 60 days of exposure, a significant level of mercury accumulation was detected in all the tissues of fish exposed to the different MeHg-spiked diets as compared to the control condition (Fig. 1). Indeed, in the organs of fish from the MeHg condition, the concentrations measured in the brain, the liver, and the skeletal muscle were respectively 215-, 255- and 64-fold higher than those measured in control fish (Supplementary Table 3).

In the tissues of fish fed food containing MeHg and supplemented with the *P. reticulata* extract, a significant accumulation was observed as compared to control fish (236-, 128-, and 64-fold higher in the brain, the liver and the skeletal muscle respectively Supplementary Table 3). In these fish, the levels of accumulated mercury in the brain and the skeletal muscle were similar to those measured in the MeHg exposure condition (Fig. 1). However, this combined exposure induced a significant 2-fold lower level of mercury accumulated in the liver compared to those measured under the MeHg condition (Supplementary Table 3).

Additionally, the levels measured in the tissues of fish fed with food containing MeHg and the *C. favosus* extract were significantly higher than those measured in the control fish (230-, 206-, and 58-fold higher in the brain, the liver, and the skeletal muscle respectively, Supplementary Table 3), and similar to those measured in fish fed MeHg-contaminated food (Fig. 1).

Suppression of MeHg-generated oxidative stress. To evaluate the antioxidant properties of the plant extracts against mercurial toxicity, the levels of lipid peroxidation were measured using the TBARS assay in the brain, liver, and skeletal muscle of the fish. The data obtained following a 60-day exposure to MeHg-contaminated food (Fig. 2) confirmed that the mercurial pro-oxidant property with

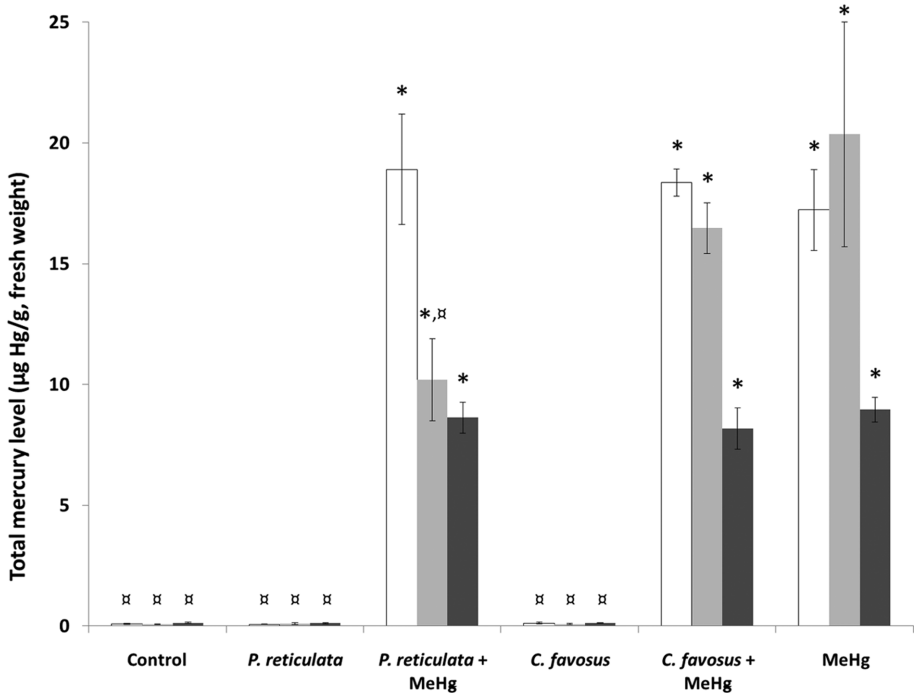

Fig. 1. Total Hg levels measured in the brain (white bars), the liver (light grey bars) and the skeletal muscle (dark grey bars) of zebrafish exposed to different diets during 60 days. Values are presented as mean ± standard deviation (SD) ($n = 3$). *Significantly different from the control group (*$p < 0.05$). ¤Statistical difference as compared to the MeHg exposure condition (¤$p < 0.05$). In the "*P. reticulata*" and "*C. favosus*" exposure conditions fish were fed with control food supplemented with 0.5% of either the *P. reticulata* or the *C. favosus* extract. In the exposure conditions "*P. reticulata* + MeHg" and "*C. favosus* + MeHg", the food was spiked with 13.5 μg MeHg/g d.w. and supplemented with 0.5% of either the *P. reticulata* or the *C. favosus* extract. In the exposure condition called "MeHg", fish were fed with contaminated food containing 13.5 μg MeHg/g d.w.

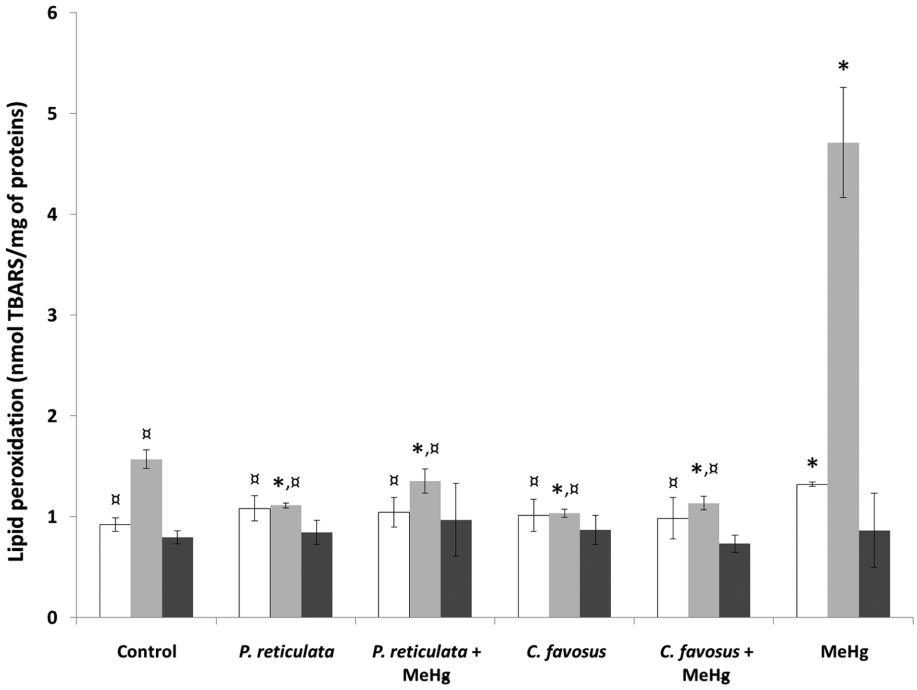

Fig. 2. Assessment of the lipid peroxidation in the brain (white bars), the liver (light grey bars) and the skeletal muscle (dark grey bars) of zebrafish exposed to different diets during 60 days. Values are presented as mean ± SD ($n = 3$). *Significantly different from the control group (*$p < 0.05$). ¤Statistical difference as compared to the MeHg exposure condition (¤$p < 0.05$). In the "*P. reticulata*" and "*C. favosus*" exposure conditions fish were fed with control food supplemented with 0.5% of either the *P. reticulata* or the *C. favosus* extract. In the exposure conditions "*P. reticulata* + MeHg" and "*C. favosus* + MeHg", the food was spiked with 13.5 μg MeHg/g d.w. and supplemented with 0.5% of either the *P. reticulata* or the *C. favosus* extract. In the exposure condition called "MeHg", fish were fed with contaminated food containing 13.5 μg MeHg/g d.w.

a significant 50% increase in the lipid peroxidation level in the brain and a 3-fold higher level of TBARS in the liver of fish compared to the controls (Supplementary Table 4).

The antioxidant property of the plant extracts was assessed by the supplementation of the control food by 0.5% of either the *P. reticulata* extract or the *C. favosus* extract. Indeed, the levels measured in the livers of these fish pointed out a significantly reduced basal level of lipid peroxidation by 30 and 34% compared to the controls after treatment with *P. reticulata* and *C. favosus* extracts, respectively (Supplementary Table 4). After spiking the vegetal extract-supplemented food with MeHg, not only did both *P. reticulata* and *C. favosus* extracts neutralize the oxidative stress generated by the MeHg uptake with observed 3-fold decreased levels of MDA in liver, but also maintained an MDA level below 14 and 27% of that of the control fish liver, respectively (Fig. 2). In the brain of these fish, the MeHg-induced lipid peroxidation was suppressed by both extracts. The TBARS levels measured in this organ were significantly lower than those observed in the MeHg-exposed fish were (20 and 25% decreased values after treatment with food containing MeHg and *P. reticulata* or *C. favosus* extract, respectively; Supplementary Table 4), and similar to those measured in the controls.

Neuroprotective potential of plant extracts. The mercurial neurotoxicity and neuroprotective potential of both plant extracts have been evaluated by the assessment of the specific initial activity of the AChE in the brain and skeletal muscle of fish fed with the different diets (Fig. 3). The results obtained following supplementation of the control food with 0.5% the *P. reticulata* extract (Fig. 3) pointed out a significant increase of the AChE activity, with a 26 and 34% higher activity both in the brain and the skeletal muscle of fish as compared to controls (Supplementary Table 5). In contrast, food supplementation with 0.5% of the *C. favosus* extract (Fig. 3) also stimulated the AChE activity, but this was only significant in the skeletal muscle with a 40% increase compared with the controls (Supplementary Table 5).

Following a 60-day exposure to MeHg-contaminated food (Fig. 3), the mercurial neurotoxic effects were highlighted by a significant 14% and 36% decrease of the AChE activity in the brain and skeletal muscle of fish as compared to the control condition (Supplementary Table 5).

The neuroprotective potential of the plant extracts was highlighted by their suppression of the mercurial neurotoxicity (suppression of the inhibition of the AChE activity). On the one hand, supplementation of the MeHg-

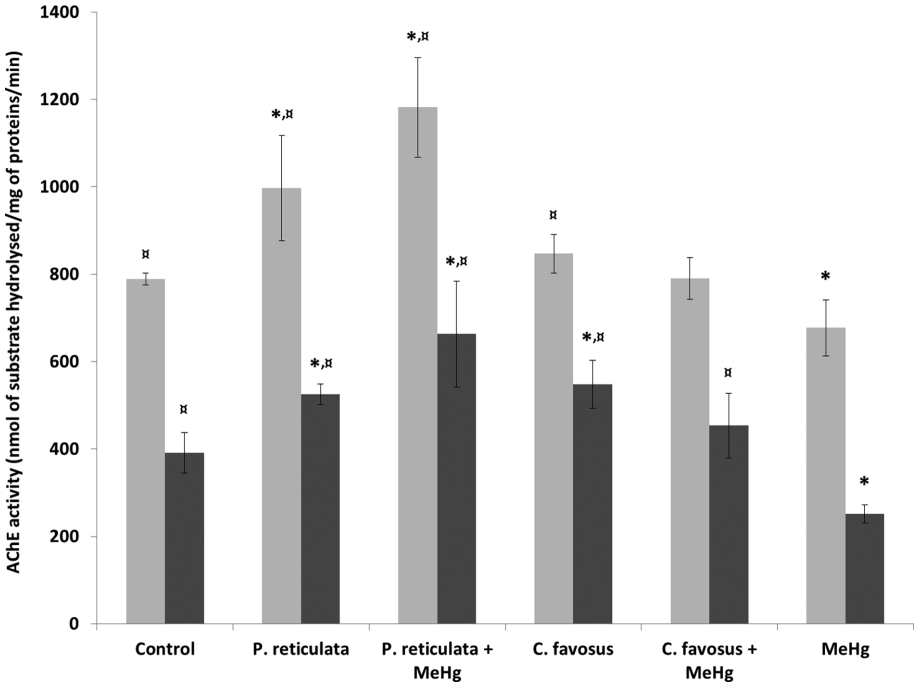

Fig. 3. Quantification of the specific initial activity of the AChE in the brain (light grey bras), and the skeletal muscle (dark grey bars) of zebrafish exposed to different diets during 60 days. Values are presented as mean ± standard error of mean (SEM) ($n = 3$). *Significantly different from the control group (*$p < 0.05$). ¤Statistical difference as compared to the MeHg exposure condition (¤$p < 0.05$). In the "*P. reticulata*" and "*C. favosus*" exposure conditions fish were fed with control food supplemented with 0.5% of either the *P. reticulata* or the *C. favosus* extract. In the exposure conditions "*P. reticulata* + MeHg" and "*C. favosus* + MeHg", the food was spiked with 13.5 μg MeHg/g d.w. and supplemented with 0.5% of either the *P. reticulata* or the *C. favosus* extract. In the exposure condition called "MeHg", fish were fed with contaminated food containing 13.5 μg MeHg/g d.w.

contaminated food by 0.5% of the *P. reticulata* extract provided the best neuroprotection (Fig. 3) since the AChE activity in both the brain and skeletal muscle of fish remained higher than that corresponding measurements in the control fish (50 and 69%, respectively; Supplementary Table 5). On the other hand, the addition of *C. favosus* extract to the MeHg-contaminated food suppressed the significant mercurial neurotoxicity in the skeletal muscle (Fig. 3) and levels of AChE activity were 80% higher than those measured after exposure to MeHg alone and equivalent to those in the control muscle (Supplementary Table 5).

Expression of sod1 and mt2 antioxidant and AChE-encoding genes in response to MeHg and plant extract-containing diets. The effects of 60-day exposure to the different diets on the relative expression of three selected genes were evaluated in the brain, liver and skeletal muscles of the fish (Table 3). Two are involved in the neutralization of the superoxide radical anion and maintenance of the redox balance, *sod1*, and *mt2*, encoding the cytoplasmic copper-zinc superoxide dismutase and the second isoform of metallothionein. The third gene was that of AChE, *ache*.

In the MeHg-fed fish, the *mt2* gene was significantly repressed 9-, 4- and 5-fold in the brain, liver, and muscle, respectively (Table 3, Supplementary Table 6). The effect of MeHg on *mt2* gene repression was reversed by both natural extracts, and even an overexpression was observed in the three tested tissues when *P. reticulata* extract was combined with MeHg, with a significant 4-, 24-, and 4-fold increased expressions compared to that of the control in the brain, liver, and muscle, respectively. The addition of *C. favosus* extract to food spiked with MeHg caused a significant 14-fold increase in expression of the *mt2* gene.

The exposure to MeHg triggered a significant 14- and 2.5-fold downregulation of the *sod1* gene expression in the liver and muscle, respectively. Supplementation of the MeHg-spiked food with either one of the natural extracts restored the level of *sod1* gene expression to a level that was similar to that of the control in the liver and muscle.

In the muscle, exposure to MeHg triggered a significant 5-fold downregulation of *ache* gene. The supplementation of MeHg-spiked food with either one of the natural extracts reversed the repression, resulting in a level of expression of *ache* gene similar to the control. Whereas the MeHg exposure did not induce a differential *ache* gene expression in the brain, supplementation of the MeHg-spiked food with *P. reticulata* and *C. favosus* extracts triggered a

Table 3. Relative genes' expression[a] reported in the brain, the liver and the muscle of zebrafish fed with different diets during 60 days. Results are presented as mean ± SEM ($n = 5$)

Tissue and gene	Control	*P. reticulata*[b]	*P. reticulata* + MeHg[c]	*C. favosus*[b]	*C. favosus* + MeHg[c]	MeHg[d]
Brain						
mt2	$(103 \pm 20) \times 10^{-1}$¤	$(60 \pm 21) \times 10^{-1}$¤	$(430 \pm 82) \times 10^{-1}$*,¤	$(101 \pm 40) \times 10^{-1}$¤	$(85 \pm 42) \times 10^{-1}$¤	$(12 \pm 4) \times 10^{-1}$*
sod1	$(6.9 \pm 0.5) \times 10^{-1}$¤	$(6.0 \pm 1.0) \times 10^{-1}$¤	$(131 \pm 26) \times 10^{-1}$*	$(14 \pm 5) \times 10^{-1}$	$(150 \pm 50) \times 10^{-1}$*	$(190 \pm 170) \times 10^{-1}$*
ache	$(4.2 \pm 1.5) \times 10^{-1}$	$(3.9 \pm 2.1) \times 10^{-1}$	$(58 \pm 12) \times 10^{-1}$*,¤	$(6.8 \pm 1.4) \times 10^{-1}$¤	$(19 \pm 5) \times 10^{-1}$*,¤	$(3.4 \pm 0.5) \times 10^{-1}$
Liver						
mt2	10 ± 0.3¤	6 ± 0.8¤	240 ± 70*,¤	13 ± 4.2¤	140 ± 80*,¤	2.4 ± 0.05*
sod1	24 ± 18¤	16 ± 9¤	18 ± 11¤	20 ± 6¤	29 ± 17¤	1.7 ± 0.3*
Muscle						
mt2	1 ± 0.4¤	7.7 ± 4.7¤	4 ± 0.2*,¤	1.9 ± 0.9¤	1.1 ± 0.1¤	0.2 ± 0.1*
sod1	$(4.9 \pm 0.8) \times 10^{-1}$¤	$(9.0 \pm 3.1) \times 10^{-1}$¤	$(9 \pm 2.9) \times 10^{-1}$¤	$(18 \pm 11) \times 10^{-1}$¤	$(2.2 \pm 0.5) \times 10^{-1}$	$(2 \pm 0.3) \times 10^{-1}$*
ache	1 ± 0.4¤	2 ± 0.1¤	2 ± 0.2¤	2 ± 0.3¤	0.9 ± 0.07¤	0.2 ± 0.07*

[a]Gene expression was calculated using the $2^{\Delta Ct}$ method and the β-actin as reference gene.
[b]Food supplemented by 0.5% of either the plant extract of *P. reticulata* or that of *C. favosus*.
[c]Food containing 0.5% of either the plant extract of *P. reticulata* or that of *C. favosus* and 13.5 μg MeHg/g (d.w.).
[d]Fish fed with MeHg contaminated food (13.5 μg MeHg/g d.w.).
Significant difference as compared to the control condition ($p < 0.05$). ¤Significantly different from the MeHg condition (¤$p < 0.05$).

significant 14- and 5-fold increased expression of those genes, respectively.

DISCUSSION

The protective action of both extracts has been demonstrated, for the first time, in yeast exposed to $CdCl_2$. The Δ*ycf1* mutant strain of *S. cerevisiae* clearly demonstrated a hypersensitivity to cadmium exposure because the wild-type yeast strain resists up to 100 μM Cd(II) (28) and up to 500 μM of Cd(II) when it overexpresses the *YCF1* gene (29), whereas in the present study, the half-maximal (50%) inhibitory concentration (IC50)50 for the Δ*ycf1* mutant was 20 μM $CdCl_2$. Thus, considering the hypersensitivity to $CdCl_2$, the results obtained in the Δ*ycf1* mutant clearly demonstrated the potential protective effects of both extracts on the antioxidant defense system of the yeast. In addition, the results more precisely indicate the ability of these extracts to reverse the onset of the expression of genes involved in response to oxidative stress and genotoxic damage triggered by the exposure to $CdCl_2$. Furthermore, this was a successful first step in the selection process of putative metal-protective extracts because challenging yeast with a combination of $CdCl_2$ and extracts saves time and spares animal lives. Finally, the yeast allowed the selection of a single dose of each extract to be used in zebrafish feed contaminated by MeHg.

The results presented here clearly highlight that the mercury-generated oxidative stress, exemplified by increased levels of lipid peroxidation, could be suppressed by food supplementation with 0.5% of either plant extracts. In addition, the levels could even be maintained at significantly lower levels than the basal levels. Previous studies have demonstrated the *in vivo* protective potential of plant extracts against metal-generated lipid peroxidation (13,14, 16,30,31). These results are in line with those observed in our study on the *in vivo* antioxidant property of both plant extracts. Indeed, while MeHg downregulated the *mt2* gene, both extracts suppressed the effect of mercury, and the *P. reticulata* extract even upregulation of the gene in the brain, liver, and muscle. These observations indicate that these extracts likely stimulated the antioxidant response, paralleling the complete reversal of the lipid peroxidation levels induced by MeHg in the brain and liver. In agreement with this idea, both extracts reversed the MeHg-promoted *sod1* gene repression in the liver and muscle.

The analysis of the phytochemical composition of the plant extracts used in our experiment identified the main fraction in both aqueous extracts are phenolic compounds (5,6). These compounds are particularly known to be primary antioxidants, acting as free radical scavengers (32) that delay or inhibit the propagation of lipid peroxidation (33-35). Therefore, they might be responsible for the observed neutralization of the MeHg-generated oxidative stress. The *in vitro* antioxidant effect of phenolic compounds extracted from garlic (*Allium sativum L.*) against lipid peroxidation has been shown on a homogenate of the rat kidney exposed to cisplatin (36). Additionally, the anti-lipid peroxidation property of polyphenolic compounds extracted from the bark and stem of *Mahonia leschenaultia* takeda (Berberidaceae) and five medicinal plants of the Colombian Amazonia has also been reported (37,38). Therefore, these results corroborate the hypothesis that the anti-lipid peroxidation activity of the aqueous extracts observed in our experiment could be mediated by their rich phenolic compound content.

Additionally, in the present study, the *in vivo* neuroprotective potential of the two aqueous extracts against mercurial neurotoxicity was highlighted, as evidenced by reversal of the MeHg-induced AChE enzyme inhibition. Furthermore, this finding was supported by the suppression of MeHg-induced *ache* gene repression in the muscle, and the upregulation of this gene was triggered in the brain by both extracts. The neuroprotective potential of the plant extracts against mercury-induced AChE inhibition has also been observed in rats (39) and the lobster cockroach *Nauphoeta cinerea* (13).

The concentration of accumulated Hg in the brain and muscle was not modified by treatment with *P. reticulata* and *C. favosus* extracts. However, a 2-fold reduction of the Hg concentration in the liver was observed after treatment with *P. reticulata* extract compared to animals exposed only to MeHg and could explain the protective effects of the extract. The brains and muscles of animals treated with the extract accumulated as much Hg as those of animals only exposed to MeHg did. Therefore, the weaker Hg concentration in the liver of extract-treated animals than control animals was not due to the modification of the properties of the intestinal barrier or chelation of MeHg by some compounds of the extract in the luminal part of the intestine. Rather, this observed effect was likely due to an increase in the detoxification capabilities of the liver. Indeed, when food was spiked with MeHg, the expression of the *mt2* gene in the liver was 100-fold greater in presence of *P. reticulata* extract than in its absence, and this was paralleled by a 2-fold drop in Hg concentration. Such an increase in MeHg sequestering capability might augment the cellular depuration of this toxic compound through dedicated ABC transporters. In agreement with this observation, the antioxidant Nrf2 transcription factor was shown to be stimulated in murine hepatocytes and it was necessary to explain the protective effect against MeHg generated by vegetal isothiocyanate. This eventually decreased the intracellular MeHg concentration mediated by the increased concentrations of MRP1 and MRP2 ABC transporters (40-42). Similarly, the Skn1 transcription factor, which is the homolog of the mammalian Nrf2 in *Caenorhabditis elegans*, is involved in the resistance of this nematode worm to MeHg, and the ethanolic extract of the guarana plant was shown to protect *skn-1*-mutated nematodes against MeHg (43). MeHg also increased Nrf2 activation by the downregulation of the Fyn kinase. This is induced by MeHg-mediated activation of Akt kinase, which inhibits glycogen synthase kinase 3β, leading to the retention of the Fyn kinase in the cytoplasm, and in turn, suppresses the Fyn-mediated exclusion of Nrf2 from the nucleus (44). This second intracellular signaling pathway is stimulated by MeHg and might explain why even with the presence of MeHg in the food, the protective effect of both extracts against lipid peroxidation in the liver was greater than that in the control animals (both neutralization of MeHg-induced radicals by phenolic compounds and MeHg-mediated stimulation of Nrf2).

In conclusion, in the present study, we demonstrated the *in vivo* protective potential of both *P. reticulata* and *C. favosus* aqueous extracts against mercury toxicity. These results are of great interest since the Amazonian riverside populations are still exposed to mercury poisoning through the consumption of Hg-contaminated fish (45). Indeed, these populations exhibit a high level of exposure to mercury with a mean hair mercury level, a bio-indicator of Hg exposure, which can reach up to 15 μg Hg/g in the highly exposed populations. This level of mercury represents the highest concentration reported worldwide and is associated with deleterious effects (12). The known effects of mercury exposure include nervous system damage in adults and impaired neurological development in infants and children (17). Hence, the results showing the protective potential of both aqueous extracts against MeHg in zebrafish could represent an interesting alternative strategy for the Amazonian fish-eating communities to address the impact of chronic exposure to MeHg-contaminated diets. However, the applicability of these findings is based on the assumption that the molecular mechanisms underlying MeHg toxicity and the protective properties of the extracts are similar in zebrafish and humans.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest of any kind.

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Supplementary Table 1. Gene-specific primers used for real time RT-PCR assays in yeast

Gene	Accession #	Direction	Sequence	Position of primers
RNR3	NM_001179416.1	Forward primer	5′-ATATGGCTGCTGATCGTGCC-3′	2090-2109
		Reverse primer	5′-TCGGTAAGCGCAGATGCCGC-3′	2390-2371
GSH1	NM_001181534.1	Forward primer	5′-GCCACTGGACTATGATCTTG-3′	1200-1219
		Reverse primer	5′-CACGGAATACGCAGCGTTCT-3′	1464-1445
KAR2	NM_001181468.1	Forward primer	5′-CCAGCCAAATGTCCACCCG-3′	959-977
		Reverse primer	5′-GCTGCACCGTATGCAACAGC-3′	1256-1237
PRX1	NM_001178304.1	Forward primer	5′-TTGCCACAGCACCTATTCTG-3′	92-111
		Reverse primer	5′-CGTGGGACTCAACATCTTCC-3′	370-351

Supplementary Table 2. Role, accession numbers and specific primer pairs used for the RT-qPCR analysis in zebrafish

Function	Gene	Accession number	Primer (5′-3′)
Cytoskeleton support	*β-Actin*	NM_131031	[a]AAGTGCGACGTGGACA
			[b]GTTTAGGTTGGTCGTTCGTTTGA
Ion homeostasis and heavy metals detoxification	*mt2*	AY305851	[a]TGCGAATGCGCCAAGAC
			[b]GCCCTTACACACGCACG
Response to oxidative stress	*sod1*	BC055516	[a]TGAGACACGTCGGAGACC
			[b]TGCCGATCACTCCACAGG
Neurotransmission	*ache*	NM_131846	[a]CCCGCACTGGTAATCC
			[b]GTTAAGCAGACGAGGC

[a]Forward primer; [b]Reverse primer.

Supplementary Table 3. Total mercury concentration in the brain, the liver and the muscle of fish fed with different diets during 60 days. Total mercury levels are expressed as μg Hg/g f.w. (mean ± SD, $n = 3$)

	Control	*P. reticulata*[a]	*P. reticulata* + MeHg[b]	*C. favosus*[a]	*C. favosus* + MeHg[b]	MeHg[c]
Brain	0.08 ± 0.03[¤]	0.07 ± 0.02[¤]	18.9 ± 2.28*	0.12 ± 0.04[¤]	18.4 ± 0.57*	17.2 ± 1.68*
Liver	0.08 ± 0.01[¤]	0.09 ± 0.04[¤]	10.2 ± 1.70*,[¤]	0.09 ± 0.03[¤]	16.5 ± 1.04*	20.4 ± 4.64*
Muscle	0.14 ± 0.03[¤]	0.12 ± 0.02[¤]	8.62 ± 0.63*	0.13 ± 0.01[¤]	8.18 ± 0.86*	8.96 ± 0.50*

[a]Food supplemented by 0.5% of either the plant extract of *P. reticulata* or that of *C. favosus*.
[b]Food containing 0.5% of either the plant extract of *P. reticulata* or that of *C. favosus* and 13.5 μg MeHg/g (f.w.).
[c]Fish fed with MeHg contaminated food (13.5 μg MeHg/g f.w.).
Significant accumulation as compared to the control condition ($p < 0.05$).
[¤]Significantly different from the MeHg condition ([¤]$p < 0.05$).

Supplementary Table 4. Levels of TBARS measured in the tissues of fish fed with different diets during 60 days. The data corresponds to the concentrations expressed as nmol of TBARS/mg of proteins (mean ± SD; $n = 3$)

	Control	*P. reticulata*[a]	*P. reticulata* + MeHg[b]	*C. favosus*[a]	*C. favosus* + MeHg[b]	MeHg[c]
Brain	0.92 ± 0.07[¤]	1.09 ± 0.12[¤]	1.05 ± 0.15[¤]	1.02 ± 0.16[¤]	0.99 ± 0.20[¤]	1.32 ± 0.02*
Liver	1.57 ± 0.09[¤]	1.11 ± 0.02*,[¤]	1.35 ± 0.12*,[¤]	1.03 ± 0.04*,[¤]	1.14 ± 0.07*,[¤]	4.71 ± 0.55*
Muscle	0.80 ± 0.06	0.85 ± 0.12	0.97 ± 0.36	0.87 ± 0.14	0.73 ± 0.08	0.87 ± 0.37

[a]Food supplemented by 0.5% of either the plant extract of *P. reticulata* or that of *C. favosus*.
[b]Food containing 0.5% of either the plant extract of *P. reticulata* or that of *C. favosus* and 13.5 μg MeHg/g (f.w.).
[c]Fish fed with MeHg contaminated food (13.5 μg MeHg/g f.w.).
Significant accumulation as compared to the control condition ($p < 0.05$). [¤]Significantly different from the MeHg condition ([¤]$p < 0.05$).

Supplementary Table 5. Values measured for the initial specific activity of the AChE in the tissues of fish fed with different diets during 60 days. Values are expressed as nmol of substrate hydrolyzed/mg of protein/min (mean ± SD; $n = 3$)

	Control	*P. reticulata*[a]	*P. reticulata* + MeHg[b]	*C. favosus*[a]	*C. favosus* + MeHg[b]	MeHg[c]
Brain	789 ± 13[¤]	997 ± 120*,[¤]	1182 ± 114*,[¤]	847 ± 44[¤]	790 ± 48	677 ± 64*
Muscle	391 ± 46[¤]	525 ± 23*,[¤]	663 ± 121*,[¤]	548 ± 54*,[¤]	453 ± 74[¤]	252 ± 21*

[a]Food supplemented by 0.5% of either the plant extract of *P. reticulata* or that of *C. favosus*.
[b]Food containing 0.5% of either the plant extract of *P. reticulata* or that of *C. favosus* and 13.5 μg MeHg/g (f.w.).
[c]Fish fed with MeHg contaminated food (13.5 μg MeHg/g f.w.).
Significant accumulation as compared to the control condition ($p < 0.05$). [¤]Significantly different from the MeHg condition ([¤]$p < 0.05$).

Supplementary Table 6. Differential genes expression[a] reported in the brain, the liver and the skeletal muscle of zebrafish fed with different diets during 60 days as compared to the control condition

	P. reticulata[a]	*P. reticulata* + MeHg[b]	*C. favosus*[a]	*C. favosus* + MeHg[b]	MeHg[d]
Brain					
mt2	=	4	=	=	1/9
sod1	=	19	=	22	28
ache	=	14	=	5	=
Liver					
mt2	=	24	=	14	1/4
sod1	=	=	=	=	1/14
Skeletal muscle					
mt2	=	4	=	=	1/5
sod1	=	=	=	=	1/3
ache	=	=	=	=	1/5

[a]The differential expression reported correspond to the significant change of the relative gene expression in a test condition as compared to the control condition. It was calculated by dividing the relative expression of the considered gene in the test condition by its level of expression in the control condition (without extract, without MeHg).
= indicates a lack of significant change of the relative gene expression in a test condition as compared to the control condition.
[b]Food supplemented by 0.5% of either the plant extract of *P. reticulata* or that of *C. favosus*.
[c]Food containing 0.5% of either the plant extract of *P. reticulata* or that of *C. favosus* and 13.5 μg MeHg/g (f.w.).
[d]Fish fed with MeHg contaminated food (13.5 μg MeHg/g f.w.).