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In vivo protective effect of dietary curcumin in fish *Anabas testudineus* (Bloch)

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Abstract The present study describes, for the first time, the protective effect of natural curcumin in vivo in a lower vertebrate, a teleost, *Anabas testudineus* (Bloch). Two doses of curcumin 0.5 and 1% were supplemented in the 40% protein feed and fed to fish for the periods, 2 and 8 weeks. The antioxidant status, protein content, and the tissue structure in experimental fish were examined after the short-term and long-term feeding. In all the curcumin fed groups, the lipid peroxidation product, thiobarbituric acid reactive substances content either decreased or unaffected. The glutathione content increased while the antioxidant enzyme activity pattern varied with time and dose. The histological analysis also confirmed the safety of curcumin retaining the normal arrangement of hepatocytes, hepatopancreas, macrophage–melanocyte centers in *Anabas*. The improved antioxidant status and protein content suggest a favorable effect for curcumin in cultured fish.

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Introduction

Turmeric (the powdered rhizome of the plant *Curcuma longa*) has long been used as a food additive, preservative, and coloring agent in Asian countries, including China and Southeast Asia. Curcumin (diferuloylmethane) (3–4%) is responsible for the yellow color of turmeric and comprises curcumin I (94%), curcumin II (6%), and curcumin III (0.3%) (Ruby et al. 1995). A wide spectrum of biological activities is attributed to curcumin, which include anticancerous (Soudamini and Kuttan 1989; Kelloff et al. 2000), antioxidant (Elizabeth and Rao 1990), anti-inflammatory (Ruby et al. 1998), antibacterial (Allen et al. 1998), antiviral (Rasmussen et al. 2000), antifungal (Apisariyakul et al. 1995), antidiabetic (Eshrat and Ali Hussain 2002), antistress (Kato et al. 1998), hepatoprotective (Park et al. 2000), and gastro protective effects (Ramirez-Tortosa et al. 1999), based on studies conducted either in mammals or in mammalian cell lines.

Aquaculture, which uses water from the river, estuary, coastal area, is prone to external pollution and the produce (fish, prawns) can be a health risk if consumed. A wide range of chemicals is currently

used in the aquaculture industry; mainly antibiotics, therapeutic chemicals (e.g., Ivermectin, Terramycin and Romet-30), copper containing anti-fouling agents etc. (Weston, 1996). Antibiotics added to water affect organisms for which they are not intended to. Thus, aquatic animals are more prone to pollution from chemicals or their own excrement. Pollutant-stimulated reactive oxygen species (ROS) production and resultant oxidative stress have been indicated as mechanisms of toxicity in aquatic organisms exposed to pollution (Livingstone 2003). ROS attack all biological molecules, especially PUFA (poly-unsaturated fatty acids) and lead to the formation of lipid peroxidation products, collectively, the thiobarbituric acid reactive substances (TBARS) content and conjugated dienes (CD), which are toxic to the body. In order to deal with the potential dangers of ROS, cells have developed a number of defense mechanisms. These include antioxidant enzymes like superoxide dismutase (SOD; EC.1.15.1.1, converts $O2^{\cdot-}$ to H_2O_2), catalase (CAT; EC.1.11.1.6, converts H_2O_2 to water), glutathione peroxidase (GPx; EC. 1.11.1.9, detoxifies H_2O_2 and organic peroxides utilizing reduced glutathione—GSH), glutathione reductase (GR; EC.1.6.4.2), and nonenzymatic antioxidants like vitamin A, E, glutathione (GSH) etc. In the normal metabolic state of a cell, a balance exists between the generation of ROS and their quenching by antioxidants. Despite the presence of antioxidant defense systems, increased levels of oxidative damage to protein, lipid, and DNA occurs in fish with laboratory and field exposure to contaminants (Livingstone 2001). Transient increases in antioxidant enzyme activities and changes in free radical scavenger levels have been observed with exposure to contaminants, but overall, relatively little is known of the regulation of antioxidant system in fish. The same general situation of pollutant-stimulated ROS production, antioxidant defense, and oxidative damage as seen for mammals is indicated for aquatic animals. However, much less is known on many of these aspects, particularly with regard to in vivo events and the relationship of oxidative damage with disease (Livingstone 2001). Studies have shown that the consumption of food (fish, meat etc.) damaged by free radicals contains toxic peroxidation products. They can be absorbed through the gut into the systemic circulation. The accumulation of the lipid peroxidation products in the body is associated with various diseases like atherosclerosis, cancer, myopathy etc.

Artificial feeds, based mainly on feed stuffs of plant origin, are less expensive and can, with proper balance of nutrients, produce better yield. Recently, there was a report (AL-Sultan 2003) that turmeric as a feed additive enhanced the overall performance in broiler chickens. Studies on the effect of curcumin on fish tissue are scanty except for a recent report by D'Souza and Prabhu (2006) that turmeric in vitro inhibited lipid peroxidation in Somberus sombrus. Lately, in vitro hepatoprotective effect of various natural curcuminoids in fish *Anabas testudineus* (Manju et al. 2008a) and an in vivo and in vitro protective effect of a synthetic curcumin (salicylcurcumin) (Manju et al. 2008b) in the same fish were investigated in our laboratory. Despite reported benefits, in vitro studies in cultured cells have demonstrated that curcumin induces chromosomal and DNA damage (Antunes et al. 1999; Araujo et al. 1999a, b) detected by the comet assay (Kelly et al. 2001; Urbina-Cano et al. 2006). In view of above results, the present work was carried out to further ensure the long-term in vivo effects and safety of the doses of natural curcumin on liver antioxidant enzymes, lipid peroxidation, and also on histological structure of the liver in the freshwater teleost, *Anabas testudineus*.

Materials and methods

Experimental conditions

Adult fish, weighing 40 ± 5 g were collected from a local supplier captured from the rivers of Thiruvananthapuram district, Kerala, using fish nets. They were reared in large stock tanks with aerated well-water (28–30°C) and natural photoperiod 12L:12D for a month. The fish were fed ad libitum with 40% protein feed (basal feed) once daily, prepared in the laboratory (Hardy 1980). The basal feed was prepared following the square method of Hardy (1980). The components were weighed and mixed with 0.5 or 1% curcumin (powder) and made into a paste and steamed. Later, adequate amount of vitamins were added, made into pellet form, and dried under shade. Its components (Table 1) and proximate composition (Table 2) were determined earlier in our laboratory (Johnson 2004).

Table 1 Proportion (g/100 g) of feed ingredients in the diet

Diet	Proportion (g%)	Protein content (g%)
Rice bran	13.24	1.15
Groundnut oil cake	36.76	8.49
Tapioca flour	13.24	0.29
Fish meal	36.76	20.07

(Johnson 2004)

Table 2 Proximate composition of the diet used in the study

Properties	Composition ± SD
Moisture (%)	12.30 ± 0.71
Protein (%)	40.02 ± 0.33
Lipid (%)	9.14 ± 0.20
Carbohydrate (%)	13.27 ± 1.01
Fiber (%)	4.81 ± 0.14
Ash (%)	2.11 ± 1.04

$n = 6$

(Johnson 2004)

Experimental diet

Curcumin was a gift from Synthite Industrial Chemicals Limited, Kochi. The curcumin present in turmeric is in fact a mixture of three closely related linear diarylheptanoids. The interest of the study was to explore the beneficial effects of curcumin in aquaculture. Therefore, at first, in vitro studies were conducted using various doses and confirmed the protective effect (Manju et al. 2008a). Later on, in vivo studies were also conducted on fish fingerlings using different doses of curcumin of which the lower doses were not effective (unpublished observation). So for our long-term study, we selected 0.5 and 1% doses of curcumin and measured the activities of hepatic and renal marker enzymes in order to assess the toxicity of the doses. Since our interest was to investigate the beneficial effects, the safe doses were fixed and conducted the studies. The experimental feeds were prepared by supplementing two different doses viz. 0.5 and 1% curcumin (by weight) to the components of the basal feed and made into a paste and steamed. Later, adequate amount of vitamins were added, made into pellet form, and dried under shade. Curcumin has been extensively investigated for its antioxidant potential, and it is now well established that curcumin is a strong antioxidant in cell lines and in mammalian models. Its in vitro protective effects on fish hepatocytes (Manju et al. 2008a) were confirmed recently in our laboratory. Therefore, no known antioxidants were used as reference for the study.

Experimental design

At the end of acclimatization, the fish weighing 40 ± 5 g were transferred to aquarium tanks (61 × 30.5 × 30.5 cm) that maintained the conditions identical to the stock tanks. The tanks were labeled A_1, A_2, A_3, B_1 B_2, B_3, C1, C_2, and C_3, with eight fish each. Tank A series fish were fed with basal feed (control), tank B series with 0.5%, and tank C series with 1% curcumin supplemented feed in each set. Each tank received accurately weighed 10% BW feed once daily in the morning hours. A short-term study was conducted for a period of 2 weeks, and a long-term study for 8 weeks.

Sample preparation

At the end of experimentation period (2 and 8 weeks), fish were fasted overnight and eight fish were selected at random from each triplicate group. They were killed by decapitation. Liver was selected for the study since it constitutes the main organ involved in detoxification and metabolism of xenobiotics. Curcumin is entirely a new compound to fish system. Therefore, at first, it was planned to evaluate effect of curcumin on the physiological state especially on the antioxidant system of the fish. The effect of various doses of curcumin on *Anabas* liver was examined by biochemical methods in the short-term study and in the long-term feeding; the effect was analyzed by histological, biochemical, and electrophoretic methods. Liver was fixed immediately in buffered formalin fixative for histology. For biochemical analysis, the tissue was collected in ice-cold containers and washed with saline and the tissues (100 mg) were homogenized using MICRA-D8 homogenizer in ice-cold sucrose buffer and centrifuged at 5,000 rpm for 10 min. The supernatant was used for the enzyme assays. For the analysis of TBARS content, tissue was homogenized in the Tris–HCl buffer, centrifuged and the same fraction was taken.

Histological sampling

Liver from five randomly selected fish from each long-term fed fish were fixed in buffered formalin was washed in running water and dehydrated by passing through ethanol series (30–100%). The tissue was then cleaned and infiltrated for 2 h in molten paraffin wax for 24 h at 60°C. The blocks prepared were then sectioned in a Leica microtome at 3–4 μm thickness. The sections were taken on slides, dehydrated once again using the same alcohol series from 100 to 30% ethanol after which it was stained using Hematoxylin–Eosin (H & E) (Clark 1981). The stained sections were mounted in DPX. Light microscopic preparations were observed under Leica research microscope, and the images were captured in a Pentium IV computer using Qwin software (Leica, Jena, Germany). Ten microscopic fields (sorted randomly) of five slides with sections taken from five different fish in each group were examined and scored in a blinded fashion by a single pathologist, and histometric measurements were taken. Surface area of hepatocyte (μm^2), surface area of hepatocyte nuclei (μm^2), and the number of hepatocyte nuclei per μm^2 of hepatic tissue [hepatocyte density (cells μm^{-2})] were determined using a Pentium IV computer using Qwin software (Leica, Jena, Germany). The hepatocellular evaluation was assessed according to Bernet et al. (1999).

Biochemical analyses

All the chemicals used were of analytical grade and purchased from SRL, Mumbai. The secondary lipid peroxidation products were determined as the thiobarbituric acid reactive substances (TBARS) content, by the method of Nichans and Samuelsson (1968). The activities of antioxidant enzymes, superoxide dismutase (SOD) (EC.1.15.1) using the method of Kakkar et al. (1984), catalase (CAT) (EC.1.11.1.6) by Maehly and Chance (1954), glutathione peroxidase (GPx) (EC. 1.11.1.9) by the method of Lawrence and Burk (1976), and glutathione reductase (GR) (EC.1.6.4.2) by the method of David and Richard (1983) were determined. Glutathione content (GSH) was assayed according to Benke and Cheevar (1974). Protein was estimated with bovine serum albumin (BSA) as standard (Bradford 1976). Absorbance was measured using a UV–visible spectrophotometer (UV-1601, Shimadzu, Japan).

Electrophoretic analyses—activity staining for SOD, CAT, GPX, and GR

Activity staining for SOD, CAT, GPx, and GR—Analytical polyacrylamide gel was performed according to a modified procedure. (Gabriel 1971). It was performed using a 10% native acrylamide slab gel in Tris–Glycine buffer, pH 8.3. The samples were mixed with 2× sample buffer without reducing agents. The concentration of protein loaded at each lane was same, and a constant voltage of 100 V was applied. Natural curcumin-treated samples (0.5%) were run along with the control for comparison. After electrophoresis, the SOD activity was visualized by the modified photochemical method of Beauchamp and Fridovich (1971). The gel was first soaked in 0.1% TEMED, 1.225 mM nitro blue tetrazolium solution and 28 μl riboflavin, and incubated in dark for 40 min. After incubation, the gel was transferred into 0.1% TEMED solution. After briefly washing, it was illuminated with a light box with an intensity of 30 $\mu Es^{-}lm^{-2}$ for 15 min to initiate photochemical reaction. After exposure, achromatic bands corresponding to SOD activity appeared on a dark blue back ground. For catalase activity staining (Sun et al. 1988), the gel was first rinsed in distilled water three times and then incubated in 0.003% hydrogen peroxide (H_2O_2) for 10 min. The gel was then stained with 2% ferric chloride and 2% potassium ferricyanide. When achromatic bands demonstrating catalase activity appeared in a green background, the staining solution was replaced and washed with distilled water. For GPx (Mishra and Fridovich 1977), the gels were stained in a solution containing 2 mM dianisidine, 10 mM potassium phosphate buffer at pH 7.2 for 1 h followed by 15 min incubation in 0.1 mM H_2O_2. Brown bands against pale yellow background were considered GPx. The gel was placed in a freshly prepared dye solution (3.4 mM GSSG, 0.36 mM NADPH, 0.052 mM dichlorophenol indophenol, 1.1 mM MTT, prepared in 250 mM Tris, pH 8) for GR activity. GR activity appeared as purple precipitate in the gel. All the activity gels were scanned, and densities were determined using Bio-Rad gel quantitation system and quantity one software.

Statistical analyses

The enzyme and the histometric data were statistically analyzed by one-way analysis of variance (ANOVA), and the protein, MDA, and GSH were analyzed by two-way ANOVA at 3, 2, and 8 weeks using the SPSS setup. The results were expressed as mean ± SE of eight animals. The significant difference among means was determined by Duncan's multiple range test (Duncan 1955) at the level, $P < 0.05$.

Results

Histological analyses

Histopathological investigations have proved to be a sensitive tool to detect direct effects of chemical compounds within target organs of animals in laboratory experiments and field animals. The present study demonstrated that the liver of both the control and experimental fish exhibited a normal structure, and there were no pathological abnormalities. The liver of control fish was formed of compactly arranged hepatocytes, a few blood capillaries, and hepatopancreas. Bile canaliculi were prominently seen. Melano-Macrophage (MM) is a special category of macrophage also present in the hepatic parenchyma, usually seen in the vicinity of hepatic arteries, portal veins, or bile ducts and concentrated as melanomacrophage centers (MMC). Numerous such patches were seen in both the control and the treated groups. Exocrine pancreatic tissue occurs around the major portal vessels and collectively called the hepatopancreas were seen abundantly. MMCs mainly occur in the vicinity of hepatopancreas. In the curcumin-treated fish, there were no pathological abnormalities with hepatocytes presenting a homogeneous cytoplasm and a large central or subcentral spherical nucleus irrespective of the dose of curcumin (Fig. 1). The hepatocyte density, hepatocyte area, and hepatocyte nuclear area were also unchanged after curcumin treatment (Fig. 2).

Biochemical analyses

Short-term study

Total protein increased in all the curcumin fed groups. SOD activity increased in the 1% curcumin fed group. The activity of CAT increased in 1%-treated group. GPx and GR activity were unaffected. Glutathione was lowered in the 1% group. TBARS decreased in all treated groups (Table 3; Fig. 3).

Long-term study

Protein content and glutathione content increased in all the groups. TBARS content was unaffected (Fig. 3). Two-way ANOVA of protein (Fig. 3a) revealed that there was a significant effect of time on the protein content of the fish ($F = 1007.5$, $P < 0.001$). As the time period increased, the protein content also increased. Similarly, there was a dose-dependent effect of curcumin on protein concentration ($F = 111.7$, $P < 0.001$). Time and dose interaction was also significant with an F value 58 and p value 0.0001. Similarly, the effect of GSH (Fig. 3b) and MDA (Fig. 3c) was also time ($F = 70.1$, $P < 0.001$ for GSH and $F = 50.3$, $P < 0.001$for MDA) and dose ($F = 23.6$, $P < 0.001$ for GSH and $F = 8.2$, $P < 0.001$ for MDA) dependent. The time and dose interaction on GSH was significant with F value 24.2, p value 0.000, whereas that of MDA was not significant ($F = 2.17$, $P < 0.131$).

Electrophoretic analyses of antioxidant enzymes

Long-term study

Liver SOD increased in density in native gel analysis at the 0.5% (Fig. 3a). Catalase increased in the 1% curcumin-treated group when compared to the 0.5% treatment group (Fig. 3b). GPx was inhibited in the 1% treatment group (Fig. 3c), whereas GR activity was unaffected in the curcumin-treated groups (Fig. 3d).

Discussion

Curcuma longa extracts have been traditionally used for centuries as a hepatoprotective agent for liver disorders. In the present study also, the normal liver histology was unaffected after the two doses of curcumin feeding, confirming an in vivo protective effect. The results were also consistent with observations by some authors in rats, guinea pigs, monkeys, and pigs (Wahlstrom and Blennow 1978;

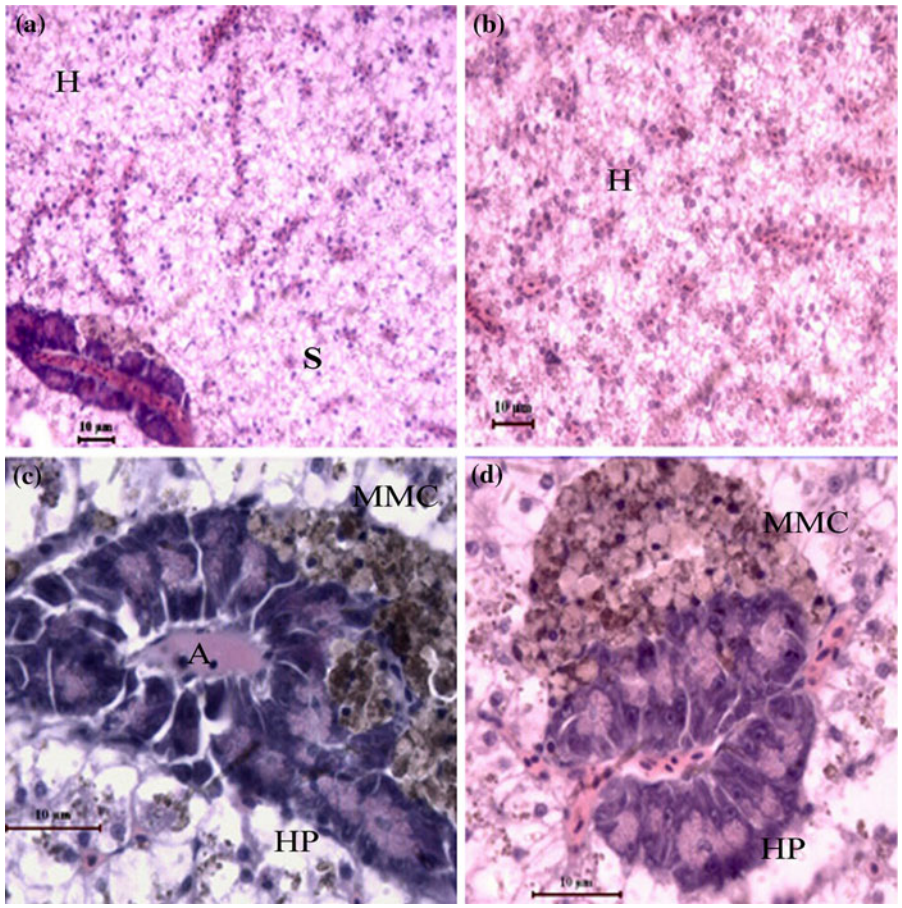

Fig. 1 Effect of curcumin on hepatocyte structure of *A. testudineus*. **a** Control (40×) **b** treated (40×) **c** control (100×) **d** treated (100×). H hepatocyte, A hepatic artery. *MMC* macrophage–melanocyte centers, *HP* hepatopancreas, *S* sinusoid

Bhavanishankar et al. 1980; Bille et al. 1985). However, there are studies which revealed that administration of turmeric extract induced hepatotoxic effects in mice and rats (Deshpande et al. 1998; Kandarkar et al. 1998). Curcumin and its analogs regained the normal histology of CCl_4-treated rat liver and caused mild sinusoidal dilation (Kamalakkannan et al. 2005). Feeding of turmeric added to diet to chicken induced dilation of bile ducts, hyperplasia of biliary epithelium, and periportal hepatocytes degeneration, which were not time and dose dependent (AL-Sultan 2003). Therefore, it can be concluded that effects of curcumin are dependent on time and dose of treatment chosen for the study (Fig. 4).

In the short-term in vivo study, lipid peroxidation product decreased in all the curcumin-treated groups, assuring their protective role in vivo too. The increase in protein in curcumin-fed group in the present study is consistent with an earlier report by AL-Sulthan (2003) in broiler chickens. SOD activity increased in the 1% group and unaffected in the 0.5% group indicating that curcumin does not always depend on the enzymatic pathway to mediate their antioxidant effect. The GPx and GR activity remain unaffected. This may be due to the increased catalase activity in the treated group, which might have depleted the substrate (H_2O_2). It has been suggested that curcumin exerts its action by maintaining the activities of antioxidant enzymes like SOD, CAT, and GPx (Pulla Reddy and Lokesh 1992). GSH, the primary intracellular free thiol fulfills many intracellular critical roles, including scavenging ROS to maintain protein bound thiols in their reduced active forms. It also serves as a cofactor for the phase II enzymes including peroxidases. The decrease in GSH content in the 1% group may be due to the presence of high

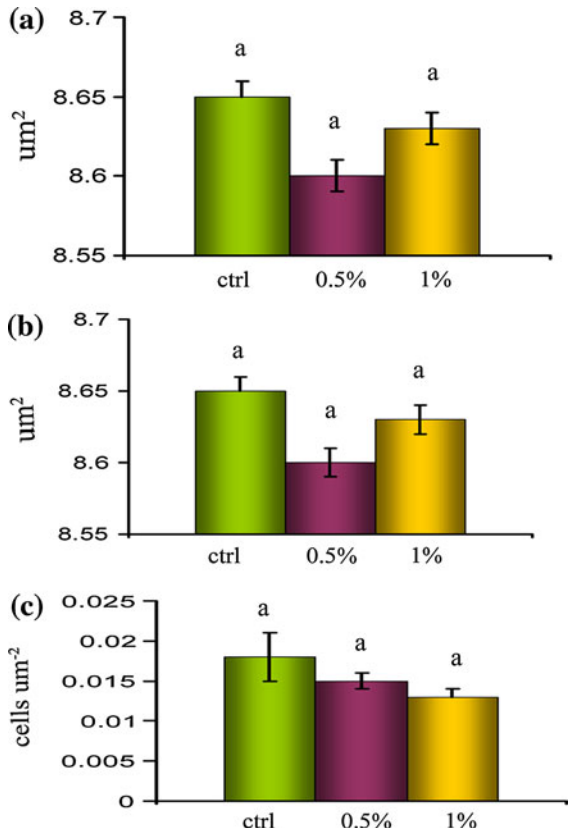

Fig. 2 **a** Effect of curcumin on surface area of hepatocyte (μm^2) **b** Effect of curcumin on surface area of hepatocyte nuclei (μm^2) **c** Effect of curcumin on hepatocyte density (cells μm^{-2}). Values are mean ± SE of 10 values. Values with same *lowercase letters* are not statistically different ($P < 0.05$) as determined by one-way ANOVA followed by Duncan's multiple range test using the SPSS setup

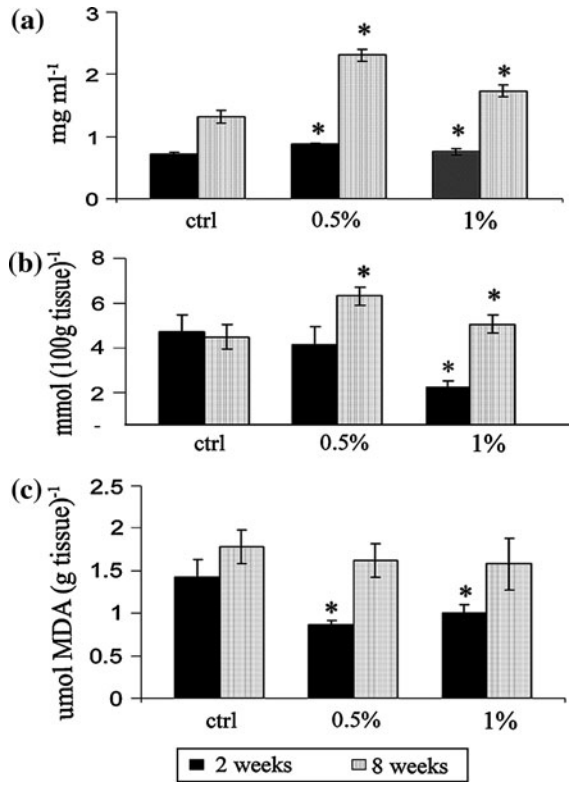

Fig. 3 **a** Effect of curcumin on protein content (mg ml^{-1}) after 2 and 8 weeks of treatment **b** Effect of curcumin on GSH content [mmol (100 g tissue)$^{-1}$] after 2 and 8 weeks **c** Effect of curcumin on TBARS content [µmol MDA (g tissue)$^{-1}$] after 2 and 8 weeks of treatment. Results expressed as mean ± SE of 8 animals. The significant difference between groups was analyzed by two-way ANOVA. $*P < 0.05$ compared with respective controls

Table 3 Effect of various doses of curcumin on fish lipid peroxidation in the in vivo short-term study

Parameters	Control	0.5% CUR	1.0% CUR
SOD (Units mg protein^{-1})	0.87 ± 0.03^{b}	0.85 ± 0.04^{b}	2.36 ± 0.08^{a}
CAT (nmoles of H_2O_2 liberated min^{-1}mg protein^{-1})	0.21 ± 0.03^{b}	0.34 ± 0.04^{b}	0.72 ± 0.08^{a}
GPx (IUmg protein^{-1})	0.85 ± 0.16^{a}	0.66 ± 0.05^{a}	0.65 ± 0.05^{a}
GR (IUmg protein^{-1})	1.26 ± 0.08^{a}	1.00 ± 0.11^{a}	1.09 ± 0.10^{a}

SOD superoxide dismutase, *CAT* catalase, *Gpx* glutathione peroxidase, *GR* glutathione reductase

Results expressed as mean ± SE of eight animals. The significant difference between groups was analyzed by one-way ANOVA

Mean values of different superscript letters (a & b) are significantly different ($P < 0.05$) as determined by Duncan's multiple range test

dose curcumin, which could also function as a nonenzymatic antioxidant similar to GSH. Even though there was no change in the SOD, CAT, and GSH in the 0.5% group, there was a significant reduction in TBARS content, indicating a direct scavenging of free radicals by curcumin.

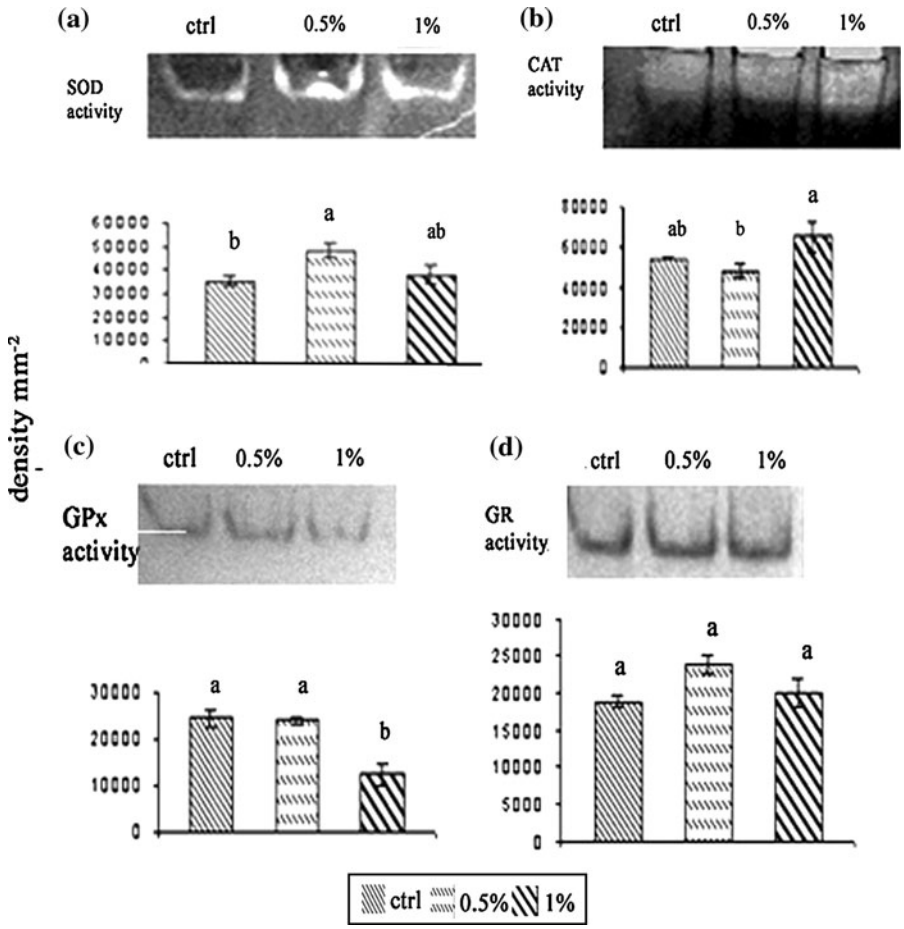

Fig. 4 **a** Effect of curcumin on superoxide dismutase activity [density (mm^{-2})] in the liver of *A. testudineus* **b** Effect of curcumin on catalase activity [density (mm^{-2})] in the liver of *A. testudineus* **c** Effect of curcumin on glutathione peroxidase activity [density (mm^{-2})] in the liver of *A. testudineus* **d** Effect of curcumin on glutathione reductase activity [density (mm^{-2})] in the liver of *A. testudineus.* Results expressed as mean ± SE of 8 animals. The significant difference between groups was analyzed by one-way ANOVA. Mean values of *different letter* headings (a and b) are significantly different ($P < 0.05$) as determined by Duncan's multiple range test, using the SPSS set up

In the long term also, there was an increase in protein concentration. But the antioxidant enzyme activity varied without affecting lipid peroxidation. The nonenzymatic antioxidant, glutathione (GSH) increased in both the treated groups. This may mean that curcumin inclusion increased cellular GSH levels likely reflecting the antioxidant and therefore, GSH sparing properties of curcumin (Rinaldi et al. 2002) as a long-term effect. Since liver is the chief organ concerned with detoxification process, availability of increased amount of GSH could improve this function. In the native gel, SOD activity increased in the 0.5% group may be due to the time-dependent effect of curcumin. CAT activity unaltered in the densitogram, may be due to the direct scavenging of H_2O_2 by curcumin. Studies have shown that curcumin prevented oxidative damage during indomethacin-induced gastric lesion, not only by blocking inactivation of gastric peroxides but also by direct scavenging of H_2O_2 and the hydroxyl radical (Halliwell and Gutteridge 1990; Bandyopadhyay et al. 1999). Another mechanism by which curcumin protects oxidative stress in endothelial cells is by induction of heme oxygenase-1 (Motterlini et al. 2000).

In conclusion, the present study confirms the protective effect of curcumin in a sub-mammalian vertebrate group that was investigated in vitro recently in our laboratory. A time- and dose-dependent effect of curcumin on fish lipid peroxidation is confirmed, as has been reported by Ambegaokar et al. (2003), Swarnakar et al. (2005), Ahuja et al. (2006) and Manju et al. (2008a, b). Curcumin may directly scavenge free radicals, stimulate antioxidant enzymes pathway, and increases the antioxidant contents in the cells. It uses different pathways to bring about its effect. More than eighty molecular targets have been reported for curcumin so far. Even though antioxidant enzymes activity inhibited, there was no increase in the TBARS content in the liver assuring its role as a promising nontoxic feed additive in the aquaculture

field. This inhibition may be a part of maintaining the normal physiology of the curcumin-treated groups. Histological data also support the protective effect. Curcumin also facilitated growth through an increase in protein concentration, which is characteristic of true growth in fish to suggest that curcumin in very low doses can be used in the aquaculture feed in order to increase the quality and quantity of fish.

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