

## Dietary Supplementation of *Limonia acidissima* L. Fruit on *in vivo* Antioxidant Activity and Lipid Peroxidation of *Cyprinus carpio* L.

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**Abstract.** *Limonia acidissima* is a tropical fruit belonging to the family Rutaceae. The present study was undertaken to investigate the effect of dietary *Limonia acidissima* (DL) on enzymatic antioxidants SOD, GPx, GST (liver, muscle, serum) and lipid peroxidation (LPo) in *Cyprinus carpio*. The fishes were fed the basal diet supplemented with DL of varying concentrations (1.5%, 3% and 6%), at feeding rates of 5% bodyweight for an interval of 30 and 60 days. Results of the study showed, an increase in SOD, GPx and GST levels were significantly ( $p < 0.01$ ) higher at 3% DL as compared to control and the other experimental groups, respectively. Levels of lipid peroxidation (LPo) also decreased consequently. DL supplementation increased the antioxidant enzyme levels and controlled the lipid peroxidation reasonably in fishes, thus augmenting their nutritive value, rendering the quality product for consumers at large.

**Keywords:** *Cyprinus carpio*, *Limonia acidissima*, antioxidant enzymes, lipid peroxidation.

### 1. Introduction

Reactive oxygen species (ROS) like the superoxide radicals ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $OH^\cdot$ ) are continuously being formed during normal aerobic metabolism. Toxic forms of activated oxygen react with cellular components resulting in oxidation of protein, DNA damage, and as well as peroxidation of unsaturated lipids in cell membranes. Fishes and crustacean against this oxidation have built up extensive defence systems, consisting of antioxidant enzymes, endogenous antioxidants such as Glutathione peroxidase (GSH-Px), Superoxide dismutase and catalase, nutritional antioxidants, such as vitamin E and carotenoids [1]. Modern aquaculture practices the principal food additives which scavenge the lipid peroxide radicals in fish feeds and are synthetic antioxidants in nature. Among these, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and ethoxyquin (EQ) are the most utilized compounds. On the other hand, these molecules have been investigated for carcinogenicity due to their potential for toxicological and adverse health effects in both fish and fish consumers through “carry-over” processes [2]. In order to avoid these toxic effects, several natural compounds have been investigated to find valid alternatives as partial substitute of synthetic antioxidants molecules. These natural antioxidants are wide class of compounds coming mainly from spices, herbs, plant polyphenols extracts (principal antioxidant compounds) [3], [4]. Several of these antioxidants are utilized in animal nutrition for feed conservation [5], for improving animal health [6] for organic animal production and for improving the quality of final product [7].

*Limonia acidissima* L. Swingle Syn. *Feronia elephantum* Correa, *Schinus Limonia* L. (Rutaceae), is a tropical plant species, indigenous to India and locally known as elephant apple. The fruits possess anti-inflammatory, antipyretic, analgesic [8], larvicidal, antimicrobial activity, widely used in Indian folk medicine, in ayurveda for the treatment of blood impurities, leucorrhoea and in yunani medicine as diuretic

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[9]. The fruit of *L. acidissima* contains flavanoids, phytosterols, glycosides, saponins, tannins, coumarins, triterpenoids, carbohydrates, vitamins and amino acids. The present study was conducted to assess the antioxidative effect of supplementation of dietary *Limonia acidissima* (DL) at varying levels (1.5%, 3% and 6%) respectively, in fresh water aquaculture and to identify appropriate concentration that will effectively induce the synthesis of antioxidant enzymes (Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Glutathione-S-transferase (GST) and control MDA levels) and combat oxidative stress in serum, liver and muscle samples of *C. carpio*.

## 2. Materials and Methods

### 2.1. Fish and experimental conditions

*Cyprinus carpio* (Common carp) fingerlings (average weight of  $3.50 \pm 1.50$  g, respectively) were procured and the experimental feeding trials were conducted at Fish seed induced carp spawning centre, Tamil Nadu government Fisheries Development Corporation Limited (TNFDC) at Aliyar (Tamil Nadu, India). A total of 200 carp fingerlings were randomly assigned into 4 individual concrete tanks (6M × 4M × 2M) at a stocking density of 50 fishes per tank. Fishes were maintained under a natural photoperiod (12-h dark/12-h light). Water quality, Dissolved oxygen concentrations ( $5 \text{ mg L}^{-1}$ ), Ammonia-N and nitrite- N concentrations ( $0.25$  and  $0.20 \text{ mg L}^{-1}$ ) were monitored daily and were within acceptable limits throughout the experimental period of 60 days.

Table 1: Ingredient composition of the basal diet (dry weight).

S.No	Ingredients	CTC(g/kgdry/Wt)	CT1(g/kgdry/Wt)	CT2 (g/kgdry/Wt)	CT3 (g/kg dry Wt)
1	Rice bran	450	450	450	450
2	Groundnut oil cake	200	200	200	200
3	Maize	100	100	100	100
4	Finger millet	100	100	100	100
5	Pearl millet	100	100	100	100
6	Nutrimin super forte*	50	50	50	50
7	Test Diet	-	15	30	60

\*Nutritional value per kg: Vitamin A - 700000 IU; Vitamin D<sub>3</sub> - 140000 IU; Vitamin E - 500 mg; Vitamin B12 - 1000 mcg; Folic Acid - 100 mg; Nicotinamide - 1000 mg; Copper - 1200 mg; Cobalt - 150 mg; Iron - 1500 mg; Zinc - 3000 mg; Iodine - 325 mg; Selenium - 10 mg; Magnesium - 6000 mg; Manganese - 1500 mg; Potassium - 100 mg; Calcium - 270 gm; Phosphorus - 130 gm; Sulphur - 7.2 gm; Fluorine - 300 mg. Nutrimin super forte obtained from AROSOL PHARMACEUTICAL PRIVATE LIMITED, Saharanpur, Uttar Pradesh, India.

### 2.2. Experimental diets and feeding frequency

Four different types of diets were prepared for the experimental trial. Out of four types of feed one was the control (CTC), with no additional supplements. Dietary supplemented feed was prepared with all the ingredients of control feed supplemented with the DL at the concentration of 1.5% (CT1), 3.0% (CT2) and 6.0% (CT3) respectively. The composition is shown in Table1. The experimental diets were fed at feeding rates of 5% of body weight, twice daily.

### 2.3. Collection of blood and serum

Blood samples were collected from randomly picked fishes of each group at day 60 after anaesthetizing with clove oil (50µl/L) (Himedia Ltd, Mumbai, India) and blood was collected from the caudal vein. The blood was then transferred immediately to the vials containing heparin solution and shaken gently. For serum, fish blood was collected without anticoagulant and allowed to clot for 2 h, centrifuged ( $3000 \times g$  for 5 min) and then kept at  $-20 \text{ }^\circ\text{C}$  until use.

### 2.4. Antioxidant and Lipid oxidation parameters

#### 2.4.1. Homogenate preparation

Fish samples of liver and muscle were homogenized in 9 volumes of 20 mM phosphate buffer pH 7.41 mM ethylene diamine tetra acetic acid (EDTA) and 0.1% Triton X-100, the homogenates were centrifuged at 600 ×g to remove debris, and the resultant supernatants used directly for enzyme assays.

### 2.4.2. Superoxide dismutase activity

Total superoxide dismutase activity was assayed by measuring the inhibition of the oxygen-dependent oxidation of adrenaline (epinephrine) to adrenochrome by xanthine oxidase plus xanthine [10].

### 2.4.3. Glutathione peroxidase activity

Glutathione peroxidase was assayed by following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase [11].

### 2.4.4. Glutathione-S transferase activity

Glutathione-S transferase (GST) activity was determined by following the formation of glutathione-chlorodinitrobenzene (CDNB) adducts at 340 nm [12]. Protein content in the homogenate supernatants was determined by the Folin-phenol reagent method, according to [13] following digestion for 1 h at 60 °C in 1 M NaOH/ 0.25% SDS.

### 2.4.5. Lipid oxidation - TBARS

TBARS indicate the oxidative changes in muscle foods during frozen storage. The amount of TBARS was determined according to the method of [14]. The amounts of TBARS were expressed as mg of malondialdehyde per kg of meat using a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

## 2.5. Statistical analysis

The data were expressed as mean ± standard error (SE). Statistical analysis of data involved one-way analysis of variance (ANOVA) followed by Tukey's pair wise comparison test. Differences were considered statistically significant when  $P < 0.05$ . The INSTAT software program was used in the statistical analysis (INSTAT™, version 3, Graphpad software, San Diego, USA).

## 3. Results

### 3.1 Antioxidant and Lipid oxidation parameters

The controls (CTC) recorded the lower SOD activity in all the three samples of liver, muscle and serum than the treatment groups (CT1, CT2 and CT3) on day 30 and 60 respectively. On the other hand, significantly ( $P < 0.001$ ) highest SOD activities of *C. carpio* was found in the liver for the treatment groups (CT1, CT2 and CT3) than muscle and serum for the 3% diet of DL on day 30 and 60 respectively (Fig. 1 & 2). The SOD activity recorded for the liver was found to be  $132.41 \pm 6.18$  and  $143.57 \pm 1.34$  U/mg protein for day 30 and 60, respectively. Therefore, appropriate diet of DL influenced the SOD activities in muscle, serum and more specifically in liver.

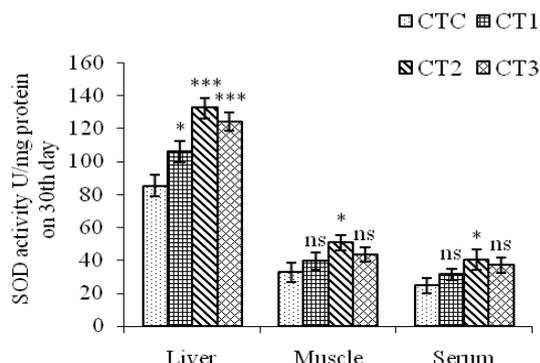


Fig 1: SOD activity of *C. carpio* on day 30

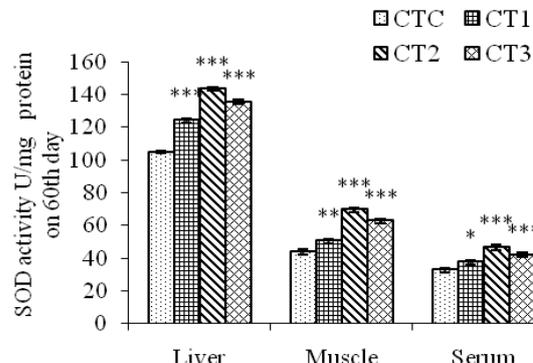


Fig 2: SOD activity of *C. carpio* on day 60

Symbol \*, \*\* and \*\*\* denotes statistically significant differences ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ ) with respect to control group.

Here was an increase in GPx activities of muscle, compared to liver and serum samples (Fig. 3 and Fig 4).

It was evident that 3% DLF supplemented diet for the treatment group CT2 was significantly different ( $P \leq 0.01$ ) and increased GPx activity of muscle and values were observed to be  $276.46 \pm 1.45$  and  $330.08 \pm 1.17$  U/mg protein and similar trend of statistically noted for the liver on day 30 and 60, respectively. Therefore, the GPx activity of tissue homogenate can be ordered as muscle > liver > serum throughout the experimental trial.

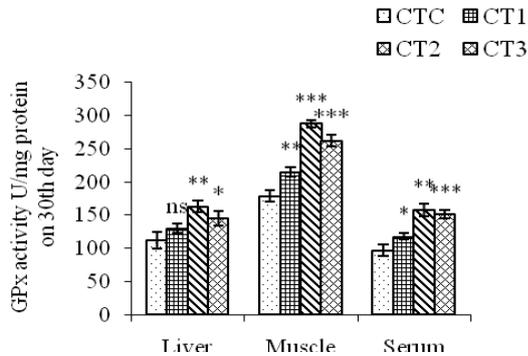


Fig 3: Gpx activity of *C. carpio* on day 30

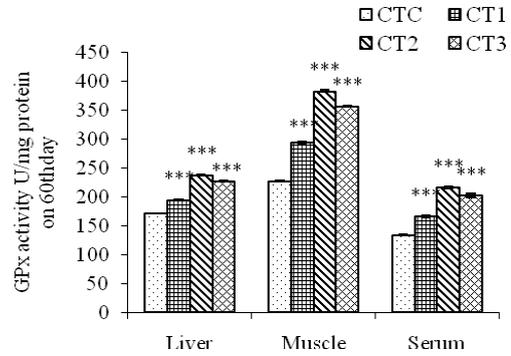


Fig 4: Gpx activity of *C. carpio* on day 60

Symbol \*, \*\* and \*\*\* denotes statistically significant differences ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ ) with respect to control group.

GST activity with varying level of DL supplementation (Fig. 5 & 6) was observed in liver, serum and muscle samples significantly different ( $P \leq 0.05$ ) on the day 30 and significantly increased ( $P < 0.001$ ) GST values were found on day 60. However, the muscle sample recorded higher activity ( $131.27 \pm 1.4$  and  $155.11 \pm 1.6$  U/mg protein) for 3% DLF diet for treatment group CT2 on day 30 and 60. The GST activity can be inferred in the following order muscle > serum > liver.

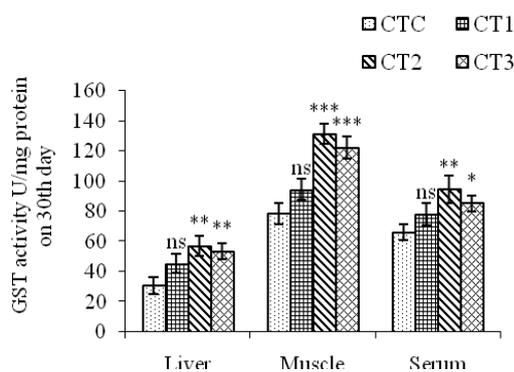


Fig 5: GST activity of *C. carpio* on day 30

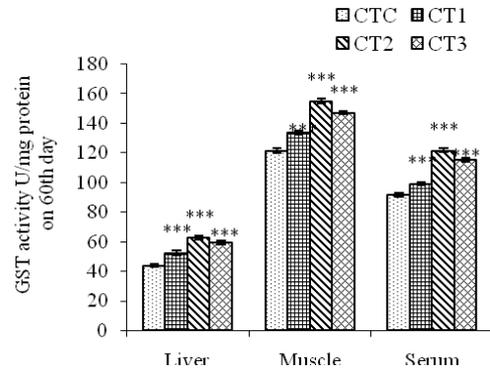


Fig 6: GST activity of *C. carpio* on day 60

Symbol \*, \*\* and \*\*\* denotes statistically significant differences ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ ) with respect to control group.

Increased levels of malonaldehyde (MDA) were noted in the liver and muscle (Fig. 7 & 8) samples of control group.

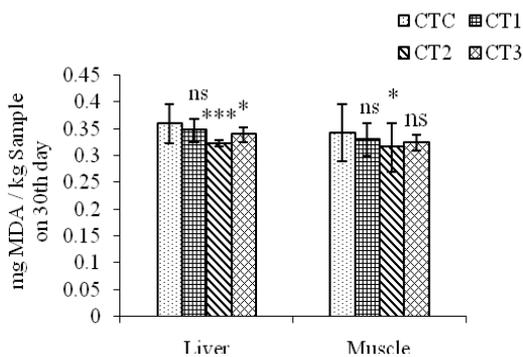


Fig 7: MDA activity of *C. carpio* on day 30

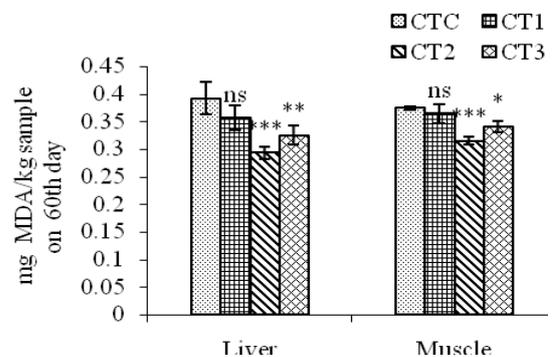


Fig 8: MDA activity of *C. carpio* on day 60

Symbol \*, \*\* and \*\*\* denotes statistically significant differences ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ ) with respect to control group.

On day 30, liver ( $0.332 \pm 0.006$ ) samples exhibited a non significant decrease in MDA levels of all experimental diets. Subsequently, on day 60, liver ( $0.293 \pm 0.01$  mg MDA/kg sample) sample exhibited a significant ( $P \leq 0.05$ ) decrease in MDA levels as compared with control groups ( $0.359 \pm 0.04$  and  $0.392 \pm 0.03$  mg MDA/kg sample, respectively.), which is the state of protection from oxidative stress levels.

#### 4. Discussion

SOD activity in liver, muscle homogenates and serum significantly increased in fish groups fed with the experimental diets. In the present study, we observed higher levels of SOD and GPx in fish fed with the 3% of DL diet compared to the control fish which may be due to enhanced nonspecific defense system, antioxidant action exerted by scavenging reactive oxygen species. These results are in agreement with [15] and [16] whose results confirm that dietary garlic supplementation significantly increased the SOD activity in *Oreochromis niloticus*, respectively. Similar results were also found in vitamin E supplemented diets on other aquatic species, such as abalone and rainbow trout [17], [18]. Further, increased SOD levels in muscle, intestine and heptopancreas in *C. carpio* var. Jian supplemented with myo-inositol (MI) was reported [19] and SOD is the first enzyme to respond against oxygen radicals and important endogenous antioxidants for protection against oxidative stress [1]. Effects of dietary antioxidants increased the GPx activity in Altanic salmon *Salmo salar* [20]. Supplementation of garlic increased the antioxidant potential by scavenging reactive oxygen species, enhancing the cellular enzymes, GPx and increased the glutathione activity in catfish after fed on diet containing selenium and vitamin E [21]. Further, reports exist on increased activity of antioxidant enzymes in hamsters, when garlic powder was fed [22], garlic oil and diallyl disulfide increased glutathione levels in RBC [23]. Glutathione dependent enzymes such as GST, GPx were able to counteract the peroxidative damage [24]. In our study, GST, GPx activities in serum, liver and muscle tissue homogenates showed significant increase in common carp supplemented with DL compared to control group. GPx activity in liver tissues of Nile tilapia exposed to Cu toxicity decreased in Nile tilapia fed on diet contained dietary antioxidant vitamin C [25]. However, there are few reports concerning the involvement of vitamins, macronutrients and probiotics in the antioxidant system as feed additives in aquatic species. It has been observed that antioxidant enzyme levels exhibit variations in dietary levels feeding [26], [27]. In the present study, the level of TBARS was significantly ( $P < 0.05$ ) lowered in fish fed with 3% DL supplemented diets than control. These results are in accordance with the findings of [17], [18], [26] who demonstrated that high levels of dietary vitamin E significantly reduced tissue lipid peroxidation in *Epinephelus coioides*, *Haliotis discus hannai* Ino, and *Oncorhynchus mykiss*, respectively.

#### 5. Conclusion

The results of the study showed that dietary DL supplementation improved the enzymatic antioxidant capacity such as SOD, GST, GPx activities and reduced the MDA levels. The lipid peroxidation of liver and muscle was prevented by the enhancement of free radical-scavenging ability in *C. carpio*. The data provides a conclusive information that DL at appropriate levels (3%) might have ideal antioxidant characteristics thus, improving the quality of the fish product.

#### 6. References

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