

# Effect of Microbially Synthesized Eicosapentaenoic Acid on Carbon Tetrachloride Induced Hepatotoxicity

Shilpa Deshpande<sup>1\*</sup>, Nandakishor Duragkar<sup>1</sup>, and Samiksha Dandekar<sup>2</sup>

<sup>1</sup>Sharad Pawar College of Pharmacy, Wanadongri, Hingna Road, Nagpur- 444011, India

<sup>2</sup>University Department of Botany, RTM Nagpur University Campus, Amravati Road, Nagpur- 440 033, India

## Abstract

**Objective:** Eicosapentaenoic Acid (EPA) from fish oil is known to have numerous health benefits including anti-inflammatory and antioxidant actions. With the intention of producing an alternative source to fish EPA, we have microbially synthesized EPA (mEPA) from rice bran oil, spectroscopically analysed and was pharmacologically evaluated. The objective of the present study was to evaluate the effect of mEPA on various hepatic enzyme biomarkers in experimentally induced hepatotoxicity.

**Methods:** Animals were divided into 7 groups of six animals each; control being treated with vehicle, another group with standard (Silymarin; 25 mg/kg per day, p.o.); 3 groups with mEPA (5, 10 and 50 mg/kg p. o.) and one with fish oil (1 g/ kg p. o.) for 15 days. The hepatotoxicity was induced in all groups except control by single dose of CCl<sub>4</sub> mixed with olive oil as vehicle in 1:1 ratio (3 ml/kg of rat body weight) on 5th day. Biochemical assays for SGOT, SGPT, ALP and total bilirubin were performed using serum samples. The histopathology of liver of all groups was carried out to compare the pathological changes.

**Results:** The serum levels of SGPT, SGPT, ALP and total bilirubin were found to be increased in the CCl<sub>4</sub> induced hepatotoxic sham control group which were significantly lowered down in the treated groups. The treatment with 50 mg/kg dose has shown maximum inhibition in enzyme levels and regenerative changes with maintained hepatic architecture compared with standard and fish oil.

**Conclusion:** Thus, microbially synthesized EPA from rice bran oil has shown promising hepato-protective effect and can fulfil the need of alternative source of EPA to fish oil.

**Keywords:** Eicosapentaenoic acid; CCl<sub>4</sub> induced hepatotoxicity; Hepatic Enzyme Biomarkers; SGOT; SGPT; ALP; Total Bilirubin; Fish Oil; Silymarin

## Introduction

Liver is involved in several vital functions and it has a great capacity to detoxify the toxic substances and synthesize useful principles. Therefore damage to the liver inflicted by hepatotoxic substances is of grave consequences. Hepatotoxicity refers to liver dysfunction or liver damage that is associated with an overload of drugs or xenobiotics. Hepatotoxicity may result not only from direct toxicity of the primary compound but also from a reactive metabolite or from an immunologically-mediated response affecting hepatocytes, biliary epithelial cells and/or liver vasculature. Hepatotoxicity can be caused by a wide variety of pharmaceutical agents, natural products, chemicals or environmental pollutants and dietary constituents [1]. Hepatotoxic chemicals cause oxidative stress, glutathione depletion and elevation of hepatic biomarker enzymes [2]. The hepatotoxins produce a wide variety of clinical and histopathological indicators of hepatic injury. Carbon tetrachloride is said to induce hepatotoxicity in rats, rabbits and humans after being metabolised to trichloromethyl free radical which causes peroxidative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes. Trichloromethyl free radicals elicit lipid peroxidation of membrane lipids in the presence of oxygen generated by metabolic leakage from mitochondria. These events lead to liver damage by loss of cell membrane integrity [1]. Liver injury can be diagnosed by certain biochemical markers like Serum Glutamic Pyruvic Transaminase (SGPT) or Alanine Amino Transferase (ALT), Serum Glutamic Oxaloacetic Transaminase (SGOT) or Aspartate Amino Transferase (AST), Alkaline Phosphatase (ALP) and bilirubin [1]. Elevations in serum enzyme levels are taken as the relevant indicators of liver toxicity. In spite of tremendous research in modern

medicine, there are hardly any specific drugs that stimulate or protect liver function or help hepatic regeneration [3].

Scientific evidence has revealed the benefits of omega-3 fatty acids. LCPUFAs like Eicosa Pentaenoic Acid (EPA) and Docosa Hexaenoic Acid (DHA) confer flexibility, fluidity and selective permeability to cellular membranes, and also produce lipid signalling molecules such as eicosanoids [4]. DHA is vital for infant growth and brain development. It also have important role in regulating gene expression, learning & memory, anxiety, mood, aggregation, depression and in epilepsy [5,6]. EPA which is the precursor of certain eicosanoids that are anti-inflammatory as compared to those derived from arachidonic acid. Therefore, various anti-inflammatory effects have been reported, such as in inflammatory bowel disease, rheumatoid arthritis, psoriasis and ulcerative colitis [7,8]. Antiproliferative and cytotoxic influences have been noted on various cell lines and inhibition of mammary tumorigenesis has been documented in animal studies [8]. EPA markedly reduces cardiovascular disease risk and improving a range of other human health conditions. Reduced serum triglyceride level is the most consistent hypolipidemic action of EPA. Its antithrombotic effect

**\*Corresponding author:** Shilpa Deshpande, Sharad Pawar College of Pharmacy, Wanadongri, Hingna Road, Nagpur- 444011, India, Tel: +09422443171; E-mail: [shilpa18@hotmail.co.in](mailto:shilpa18@hotmail.co.in)

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can be attributed to altered platelet function and possible modulation of the fibrinolytic system [8,9] Some animal and human studies have shown a beneficial effect of fish LCPUFAs in non-alcoholic fatty liver disease by reducing the expression of pro-inflammatory molecules and thus can be protective in hepatotoxicity [9].

The EPA has been successfully synthesized in our laboratory by microbial transformation of Alpha Linolenic Acid isolated from Rice bran oil (mEPA) and was spectroscopically analysed [10]. This was synthesized in the view of developing an alternative source of EPA other than fish to be used in various pathological conditions including cardiovascular, CNS and hepatic diseases. Hence, objective of the present study was to provide evidence for the hepatoprotective effect of mEPA in experimentally induced hepatotoxicity.

## Materials and Methods

### Drugs and chemicals

The kits for the hepatic biomarker enzymes like SGPT, SGPT, ALP and total bilirubin were obtained from Span Diagnostics Limited, India; Autopak, Siemens and Biovision. Silymarin was obtained from Shreeji Pharma International, Vadodara, India. Other chemicals used were of analytical grade. The fish oil used as positive standard was obtained from Central India Pharmaceuticals, Nagur, India and contained 25 % of omega-3 fatty acids as stated. The mEPA synthesized was having 89.32 % purity.

### Experimental animals

Adult male wistar rats weighing around 150-200 gm were taken and maintained under normal laboratory conditions of temperature  $24 \pm 2^\circ\text{C}$  and natural light-dark cycle and had free access to drinking water and standard pellet diet. The protocols of animal studies were approved by Institutional Animal Ethical Committee (Reg. No. 536/02/ CPCSEA, dated 20.01.02).

### Methods

Animals were divided into 7 groups of six animals each; control being treated with vehicle, another group with standard (Silymarin; 25 mg/kg per day, p.o.) 3 groups with mEPA (5, 10 and 50 mg/kg p. o.) and one with fish oil (1 gm/ kg p. o.) for 15 days. The hepatotoxicity was induced in all groups except control by single dose of  $\text{CCl}_4$  mixed with olive oil as vehicle in 1:1 ratio (3 ml/kg of rat body weight) on 5<sup>th</sup> day. Biochemical assays for SGOT, SGPT, ALP and total bilirubin were performed using serum samples. The histopathology of liver of all groups was carried out to compare the pathological changes.

### Toxicity studies

Toxicity study was carried out in rats as per OECD guidelines [11]. The dose level was selected from one of the four fixed levels 5, 50, 300, 2000 mg/kg body weight p. o. The rats were continuously observed for their mortality and behavioral response for 48 h and thereafter once in day for 14 days.

### Treatment

Animals were divided into seven groups of six animals each and were treated for 15 days as follows:

Group 1: control with vehicle

Group 2: Sham control (hepatotoxicity induced)

Group 3: Standard drug Silymarin (25 mg/kg, p. o.)

Group 4: mEPA (5 mg/ kg, p. o.)

Group 5: mEPA (10 mg/ kg, p. o.)

Group 6: mEPA (50 mg/ kg, p. o.)

Group 7: Fish oil as positive control for EPA (1 gm/ kg, p. o.)

**Induction of hepatotoxicity:** In the study animals, hepatic injury was induced in all groups except control on 5<sup>th</sup> day of treatment by single i. p. dose of  $\text{CCl}_4$  mixed with coconut oil as vehicle in 1:1 ratio (3 ml/kg of rat body weight) [12] Respective drug treatments were continued upto 15 days.

**Collection of blood samples and assessment of hepato protective activity:** After 15 days of experimental period blood sample were collected individually by retro-orbital puncture for all the animals after overnight fasting and serum was separated. It was analyzed for the biochemical markers of liver damage and dysfunction. These included SGPT, SGOT, ALP and total bilirubin.

**Histopathology:** The animals were sacrificed, liver samples were isolated and the individual weights of the livers were estimated. For histopathological study, liver tissue was fixed in 10% formalin. Liver lobe were sectioned, which were embedded in paraffin after being dehydrated in alcohol and subsequently cleared with xylene. Five-micrometer thick serial histological sections were obtained from the paraffin blocks and stained with hematoxylin and eosin. The sections were examined under light microscope and photomicrographs were taken [13].

### Estimation of hepatic enzyme markers

**Estimation of SGPT and SGOT:** The method given in the kits provided by Autopak, Siemens were used for the estimation of SGPT and SGOT. In a test tube, 1 ml of respective reconstituted reagent and 100  $\mu\text{l}$  were added and the absorbance was read at 340 nm using the specified parameters in the kit. There is a decrease in absorbance at 340 nm as NADH is converted to NAD. The rate of decrease in absorbance is measured and is proportional to SGPT / SGOT activity in the sample [14].

**Estimation of ALP:** ALP was estimated using the commercial kit provided by Biovision. Alkaline Phosphatase (ALP) catalyzes the hydrolysis of phosphate esters in alkaline buffer and produces an organic radical and inorganic phosphate. The assay uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate which turns yellow ( $\lambda_{\text{max}} = 405 \text{ nm}$ ) when dephosphorylated by ALP [15].

**Estimation of total bilirubin:** 100  $\mu\text{l}$  of reagent 1, 25  $\mu\text{l}$  of reagent 2, 500  $\mu\text{l}$  of reagent 3 provided in the kit and 100  $\mu\text{l}$  of sample were mixed and incubated at  $37^\circ\text{C}$  for 5 min. the blank was prepared without reagent 3 and absorbance was read at 580 nm. In presence of caffeine as accelerator, total bilirubin couples with diazotized sulfanilic acid formed in the first reaction to give an azo dye, the colour intensity of which is proportional to the bilirubin concentration [16].

**Statistical analysis:** All the data is expressed in Mean  $\pm$  SD. The statistical significance between more than one groups were tested by one way ANOVA using Graph Pad Prism software Version 6.04 or unless specified. The level of significance used are \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ .

## Results and Discussion

The serum levels of SGPT, SGPT, ALP and total bilirubin were found to be increased in the  $\text{CCl}_4$  induced hepatotoxic sham control group which were significantly lowered down in the treated groups.

The treatment with 50 mg/ kg dose has shown maximum inhibition in enzyme levels and regenerative changes with maintained hepatic architecture compared with standard and fish oil.

The weight of liver in CCl<sub>4</sub> induced group was significantly increased (5.78 ± 0.10 gm to 8.45 ± 0.84 gm; 146.19 %). Liver weights were significantly reduced in animals treated with 50 mg/ kg mEPA (4.20 ± 0.62 gm), silymarin (4.24 ± 0.77 g) and fish oil (4.85 ± 0.21 gm) (Table 1).

The serum levels hepatic enzyme markers were found to be increased in the CCl<sub>4</sub> induced hepatotoxic group viz. SGOT (267 ± 62.31 U/lit to 567 ± 76.24 U/lit; 212.36 %), SGPT (105.0 ± 10.92 U/lit to 283.0 ± 49.60 U/lit; 269.52 %), ALP (115 ± 14.52 U/lit to 580 ± 56.44 U/lit; 504.35 %) and total bilirubin (0.23 U/lit to 0.94 U/lit; 408.70 %).

The increased levels of hepatic enzyme markers were significantly lowered down in the treatment groups. The treatment with 50 mg/ kg dose has shown maximum inhibition (83.25 % SGOT; 84.19 % SGPT, 74.14 % ALP, 73.4 % total bilirubin) compared with standard and fish oil. The effects of 10 mg/ kg were also significant with p ≤ 0.05 whereas 5 mg/ kg dose did not exert significant effect. Although the levels were reduced with silymarin, its effect was not found statistically significant (Table 2).

Liver sections from control group showed normal hepatic architecture. In CCl<sub>4</sub> treated sham control group, degenerated and necrotic hepatocytes in centrilobular region were seen along with hepatic lesions, fatty degeneration, distortion of hepatic cords, dilated and congested central vein and other morphological signs of congestion. On examinations, liver was enlarged, pale and surface was smooth and tiny red spots were seen on its surface. The regenerative changes with maintained hepatic architecture almost similar to control were observed with 50 mg/ kg of mEPA and fish oil. Less significant changes were seen with lower doses and standard drug silymarin (Figure 1).

The liver predisposes to oxidative stress by amplifying the capacity of free radical chain reaction. An obvious sign of hepatic injury is the leakage of cellular enzymes into the plasma due to hepatocellular necrosis resulting in the disturbances in transport function of hepatocytes. When liver cells are damaged by CCl<sub>4</sub>, it causes increased enzyme levels such as SGOT, SGPT, ALP and bilirubin [2]. CCl<sub>4</sub> can affect hepatocellular calcium homeostasis and can induce hepatic damage through the formation of reactive free radicals that can bind covalently to cellular macromolecules resulting in inhibition of protein synthesis and. Overall, CCl<sub>4</sub> treatment can result in centrilobular steatosis, inflammation, apoptosis and necrosis [17].

In the present study, acute dose of CCl<sub>4</sub> was employed to avoid permanent hepatic damage. Omega-3 fatty acids are known to increase antioxidant status when given in various pathological conditions. In another study in our laboratory, mEPA has reduced oxidative stress and increased levels of antioxidant markers like superoxide dismutase, catalase and peroxidase [18]. Possible mechanism of protection exerted by mEPA in CCl<sub>4</sub> induced liver damage may include membrane stabilizing action on hepatocytes. It could serve as antioxidant that makes the hepatocytes that makes the hepatocytes less susceptible to the damaging action of noxious chemicals. Additionally, present study has shown hepatoprotection at 50 mg/kg dose of pure mEPA as compared to omega-3 fatty acid containing fish oil supplementation at 1 gm/kg dose [2].

## Conclusion

Thus, microbially synthesized EPA from rice bran oil has shown

Group	Liver Weight (gm)
Control	5.78 ± 0.10
CCl <sub>4</sub> treated Sham control <sup>a</sup>	8.45 ± 0.84 <sup>**</sup>
Standard Silymarin (25 mg/ kg)	4.24 ± 0.77 <sup>*</sup>
mEPA (5 mg/ kg)	6.56 ± 0.32
mEPA (10 mg/ kg)	5.50 ± 0.96
mEPA (50 mg/ kg)	4.20 ± 0.62 <sup>**</sup>
Fish oil (1 gm/ kg)	4.85 ± 0.21 <sup>*</sup>

Table 1: Effect of mEPA, standard drug silymarin and fish oil on liver weight of rats.

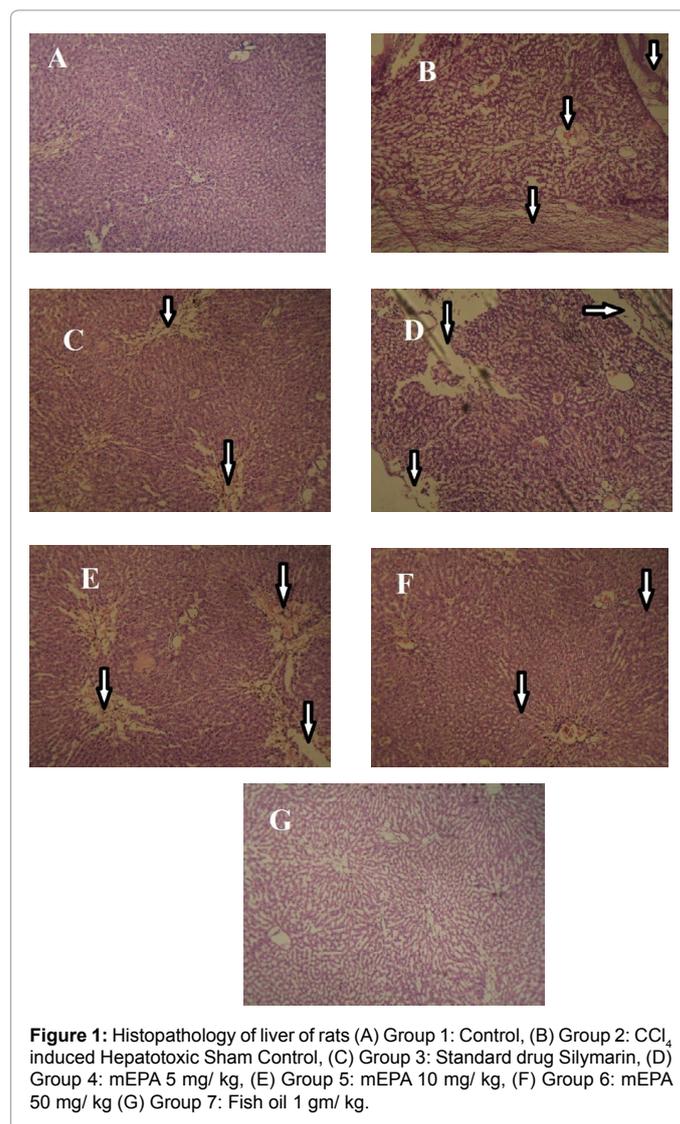


Figure 1: Histopathology of liver of rats (A) Group 1: Control, (B) Group 2: CCl<sub>4</sub> induced Hepatotoxic Sham Control, (C) Group 3: Standard drug Silymarin, (D) Group 4: mEPA 5 mg/ kg, (E) Group 5: mEPA 10 mg/ kg, (F) Group 6: mEPA 50 mg/ kg (G) Group 7: Fish oil 1 gm/ kg.

promising hepatoprotective effect and can fulfil the need of alternative source of EPA to fish oil. To conclude, mEPA treatment has induced a complete reversion of liver toxicity induced by CCl<sub>4</sub> as evident from its effect on hepatic biomarkers and histopathology at very low doses as compared to the requirement of fish oil or omega-3 fatty acid supplements, suggesting its significant hepatoprotective capability. These results propose the therapeutic application of microbially synthesized EPA and suggest that mEPA can be a good alternative source to fish LCPUFAs. However, further clinical studies are to be done to prove its clinical efficacy.

Groups	SGOT (U/ L)	SGPT (U/ L)	ALP (U/ L)	Total Bilirubin (U/ L)
Control	267 ± 62.31	105.0 ± 10.92	115 ± 14.52	0.23 ± 0.002
CCl <sub>4</sub> treated Sham control <sup>a</sup>	567 ± 76.24 <sup>***</sup>	283.0 ± 49.60 <sup>*</sup>	580 ± 56.44 <sup>**</sup>	0.94 ± 0.12 <sup>**</sup>
Standard (Silymarin; 25 mg/kg)	303 ± 58.02	180.0 ± 13.34	156 ± 25.02 <sup>*</sup>	0.32 ± 0.045 <sup>*</sup>
mEPA (5 mg/ kg)	133 ± 31.70 <sup>*</sup>	106.0 ± 17.03	500 ± 45.10	0.65 ± 0.740
mEPA (10 mg/ kg)	132 ± 1.29 <sup>*</sup>	100.0 ± 27.95 <sup>*</sup>	375 ± 18.34 <sup>*</sup>	0.42 ± 0.009 <sup>*</sup>
mEPA (50 mg/ kg)	95 ± 44.16 <sup>**</sup>	44.75 ± 3.30 <sup>*</sup>	150 ± 24.33 <sup>**</sup>	0.25 ± 0.012 <sup>**</sup>
Fish oil (1 g/ kg)	205 ± 29.60 <sup>*</sup>	114.3 ± 4.35 <sup>*</sup>	149 ± 15.66 <sup>*</sup>	0.36 ± 0.301 <sup>*</sup>

N = 6; Values are expressed in Mean ± SD <sup>a</sup> control group compared with sham control group; treated groups are compared with sham control group. <sup>\*</sup> p ≤ 0.05; <sup>\*\*</sup> p ≤ 0.01; <sup>\*\*\*</sup> p ≤ 0.001; <sup>\*\*\*\*</sup> p ≤ 0.0001.

SGOT: Serum Glutamate Oxaloacetate Transaminase, SGPT: Serum Glutamate Pyruvate Transaminase, ALP: Alkaline Phosphatase

**Table 2:** Effect of mEPA, standard drug silymarin and fish oil on liver enzyme markers.

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