CHAPTER VI

EFFECT OF SPINY EEL FISH OIL ON LIVER MARKER ENZYMES AND GENOTOXICITY IN WISTAR RATS MODEL OF ETHANOL INDUCED LIVER

Introduction

Liver and Alcohol

Alcohol is one of the main causes for end-stage liver disease worldwide. Liver is the most important organ, which plays a vital role in regulating various physiological processes in the body. It is involved in several vital functions, such as metabolism, secretion and storage. It has great capacity to detoxicate toxic substances and synthesize useful principles (Clinard and Ouazrir, 2002). Liver diseases are mainly caused by toxic chemicals, excessive consumption of alcohol, infections and autoimmune disorders. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damage.

Alcohol Induced Liver Injury (ALI) and disease (ALD) are the major health problems affecting a broad patient population of different gender, race, and social backgrounds Tadic et al., 2002; Barrio et al., 2004. In addition, the various rodent models of alcohol-induced liver damage (ALD) differs significantly with regard to species, animal age, growth rate, and the development of endotoxemia preceding pathology Tsukamoto et al., 1985. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences. Although liver damage is related to the amount of alcohol consumed, only 20% of those consuming>80 g per day develop chronic alcoholic liver disease (ALD) (Bellantani et al., 1997; Becker et al., 1996). One reason is that the various rodent models of ALD differ significantly with regard to animal age, nutritional status, growth rate, and the development of endotoxemia preceding pathological alterations (Arteel, 2003). Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages.

In spite of tremendous strides in modern medicine, there are hardly any drugs that stimulate liver function, offer protection to the liver from damage or help regeneration employed in traditional system of medicine for liver affections (Romagnuolo et al., 1998)
of hepatic cell Clinard and Ouazrir, 2002. Except for the combination of abstinence from alcohol and supportive care (Bouneva et al., 2003), however, there is no satisfactory therapy for alcoholic liver disease at present. The spectrum of alcoholic liver disease ranges from fatty liver to alcoholic hepatitis and ultimately to fibrosis and cirrhosis (Tuma and Sorrell, 2002). Much progress has been made in understanding the mechanisms of alcoholic liver disease, suggesting a complex pathological process that subsequent to alcohol metabolism in the liver and involves multiple types of cells, such as hepatocytes, Kupffer cells, neutrophils, endothelial cells and hepatic stellate cells (Tsukamoto and Lu, 2001).

**Liver Marker Enzymes**

Liver is the highly affected organ, primarily by toxic agents and so the study of serum enzyme activity has been found to be of great importance in the assessment of liver damage (Chattopadhyay et al., 1990). Long term alcohol consumption prolongs the inflammatory process leading to excessive production of free radicals, which can destroy healthy liver tissue (Nanji, 1994). Determination of the activity of hepatic enzymes released into the blood by the damaged liver is one of the most useful tools in the study of hepatotoxicity.

In animals and man, biotransformation or xenobiotic metabolizing enzyme systems are present in most if not all tissues, with the highest concentration found in liver Ennulat et al., 2010. Expression of these enzymes is influenced by a range of factors including diet, sex, age, environmental exposures, and most importantly, endobiotics and xenobiotics, including drugs and chemicals. Although drug-metabolizing enzyme (DME) systems are generally present at low levels in the liver, they can be rapidly and reversibly upregulated in response to endogenous or exogenous stimuli, a process known as enzyme induction. Increased alanine aminotransferase (ALT), aspartate aminotransferases (AST), alkaline phosphatase (ALP), and/or gamma glutamyltransferase (GGT) activities in liver parenchyma have been described in association with drug-induced CYP450 induction in the rat, the dog, and the human (Xu et al., 2005). The aminotransferase - Serum glutamate oxaloacetate transferase (SGOT) and serum glutamate pyruvate transferase (SGPT) are synthesized in the liver, heart and skeletal muscles. These enzymes catalyze the reversible transfer of amino group to a keto group which is of clinical importance (Chatterjee, 1995). Elevation of these enzymes indicates liver, heart, muscle or brain injury (Calbreth, 1992).
Chemicals from nature have been a part of human civilization ever since our early ancestors began exploiting natural compounds to improve and enrich their own lives (Agosta, 1996). A major part of these chemicals come from animals. Indeed, animals are therapeutic arsenals that have been playing significant roles in the healing processes, magic rituals, and religious practices of peoples from the five continents (Costa-Neto and Marques, 2000). The healing of human ailments by using therapeutics that are obtained from animals or ultimately derived from them is known as zootherapy. In India, since times immemorial, great work was done in this field and documented in works like Ayurveda and Charaka Samhita. Additionally, immense knowledge has come down to modern times through folklore as various practices became a part of tradition amongst various groups. We can find that in our rural people still use various animal products and by-products for the treatment of various diseases Mahawar and Jaroli (2007). Nearly 15–20 percent of the Ayurvedic medicine is based on animal-derived substances Unnikrisnhan (1998). Fish oil can have a therapeutic role in the treatment of marked hypertriglyceridemia (750 mg/dL).

Fish oils are the good source of omega-3 EFAs (Bays and Lansing, 1994). Fish oils containing omega-3 EFAs eicosapentacenoic acid (EPA) and docasahexaenoic acid (DHA) show positive effect in prevention and therapy of cardiovascular diseases (Korstanje et al., 1991). Since then from research unraveling the mechanism of Omega -3 fatty acids, to clinical trials in cardiovascular disease, treatments of hyperlipidemia, rheumatoid arthritis, and the prevention of preterm labor, there have been more than 7000 reports including nearly 900 human clinical trials on the study of fish oil and Omega -3 fatty acids potential health benefit from fish consumption (Glomset, 1985).

Increased intake of omega-3 polyunsaturated fatty acids (n-3 PUFA) has been shown to decrease the risk of cardiovascular events (Kris Etherton et al., 2003). The protective effect of n-3 PUFA seems to be linked in part to its cardiac antiarrhythmic properties (Christensen et al., 2001; Holguin et al., 2005; Kris Etherton et al., 2003; Leaf, 2001) Long-chain n-3 PUFA appears to act both directly (by replacing arachidonic acid as an eicosanoid substrate and inhibiting arachidonic acid metabolism) and indirectly by altering the expression of inflammatory genes through its effects on transcription factor activation (Calder, 2006). The present study was conducted to study the fatty acids composition with special emphasis on omega-3 essential fatty acid due to its importance.
from medical point of view, in oils extracted from *Mastacembelus armatus*. This research work provided a very useful data about fatty acid composition (omega-3 essential fatty acids) and opened new doors for further research in this field for the benefits of human beings.

In the present study, the extent of liver damage was assessed by determining the hepatic biochemical and liver marker enzymatic status by measuring the hepatic content of Alanine transaminase, Asparate transaminase, Acid Phosphatase, Alkaline Phosphatase, Lactate dehydrogenase and Gamma-glutamyl transferase, Albumin, Cholesterol, Bilirubin, Total Protein and liver glycogen. The oral administration of fish oil reversed the above parameters providing hepatoprotective efficacy of ethanol induced rats.

**Materials and Methods**

**Study Approval**

The study was approved by the Institutional Animals Ethics Committee (722/02/a /CPCSEA-dt 04.12.2006) at Bharathiar University, Coimbatore, Tamilnadu, India.

**Animals**

The Wister strain rats were maintained as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and regulations. Albino Wister Rats body weight of 100-120 gms were obtained from JKK college of Pharmacy, Komarapalayam, Namakkal District, Tamil Nadu, India. The animals were housed in well ventilated animal dwelling with constant 12±1 hours light and dark schedule. They were provided with standard diet and clean water and *libitum*.

**Spiny Eel Fish Oil Extraction**

*M. armatus* matured fish (Average 150 g) were collected from Cauvery River. The fish mucus was removed and washing with clean water. The fishes were dissected carefully and removed internal organs. After organ removing fish tagged with ropes dried in sunlight. Fully dried eel fishes were taken and cut in to small pieces then powder form in Powder Mill. *M. armatus* powder filled with autoclavable bags and cooked with steam used by autoclave. The cooked fish powder compressed oil extraction using Electric Pelletizer or hand Pelletizer. The Crude Eel fish oil used further Experiment.
Plate 1. Oil Extraction of Spiny eel *Mastacembelus armatus*

Figure: 1 Dry fish *M. armatus*  
Figure: 2 Dry fish cutting of *M. armatus*

Figure: 3 Dry fish Powder  
Figure: 4 Spiny Eel Oil  
Figure: 5 Commercial Cod Liver Fish Oil
Plate 2.

Figure: 1 Schematic Diagram of Fish Oil Extraction from Dried fish Powder

Figure: 2 Autoclave

Figure: 3 Pelletizer

Figure 4: Hand Pelletizer
Experimental Protocol

Animals were divided into Five Experimental groups

**Group I:** Controls

Positive Controls only standard diet

**Group-II:** Negative Controls

The animals administrated with 40% of alcohol consist of 3ml orally.

**Group-III:** 40% Alcohol and Standard Diet mixed with Cod Fish Oil (5ml/kg)

The animals administrated with 40% Alcohol and Fish Oil mixed diet given periodically thrice a day.

**Group-IV:** 40% Alcohol and Standard Diet mixed with Spiny Eel fish Oil (5ml/kg)

The animals administrated with 40% Alcohol and Oil mixed diet given thrice a day.

**Group-V:** 40% Alcohol and Standard Diet mixed with Spiny Eel fish Oil and Cod Liver Oil

The animals administrated with 40% Alcohol and (2.5ml/kg) Combined Cod, Spiny Eel Oil (2.5ml/kg) and standard mixed diet thrice a day consecutively.

1. **Experimental in Short Duration**

The animals were treated with alcohol and Fish oils and standard diet supplementation at 12 hours intervals for 30 consecutive days.

2. **Experimental in Long Duration**

The animals were treated with Fish oils and Standard diet Supplementation at 12 hours intervals for 90 consecutive days.

Each Experimental groups consists of six experimental animal models. These experimental animals were treated with the following regimens; all the treatments were constructed on the basis of adult dosage prescribed by the physicians.

**Blood Collection and Preparation of Sample for Antioxidant and Histology Analysis**

The blood sample were collected at the end of the experimental period the rats were deprived of food for overnight and the experimental animals were sacrificed by
cervical dislocation and the blood was collected by puncturing the heart of the mouse and incubated in room temperature for 30 minutes. The clots were then centrifuged for 15 minutes at 2000 rpm to separate serum and used for biochemical analysis.

The liver was removed for histopathological analysis. A part of the liver was washed with ice cold tris-buffered saline, blotted dry and 10% homogenate was prepared using tris buffered saline (pH 7.4) to follow lipid per oxidation rate, 15% homogenate were prepared using phosphate buffered saline (pH 7.0) in cold condition for the estimation of antioxidants and liver marker enzymes. 20% liver homogenate were prepared using 5% trichloroacetic acid to estimate reduced glutathione. The homogenate was centrifuged at 2000 rpm for 10 minutes and the supernatant was used for the experiment.

**Assays of activities of Aspartate transaminase (AST) and Alanine transaminase (ALT) (Reitman and Frankel’s Methods, 1957)**

**Principle**

AST catalyses the transfer of an amino group from L-asparatate to alphaketoglutarate to form oxaloacetate (unstable and gets converted into pyruvate) and L-glutamate. The pyruvate liberated reacts with 2, 4-dinitrophenyl hydrazine to from 2,4-dinitrophenyl hydrazone, which can be read at 540 nm.

The amount of oxaloacetate was measured by converting it into pyruvate by treating with aniline citrate and then by the reaction of pyruvate with 2, 4- dinitropheryl hydrazine. The brown colour produced was read at 520nm.

**Procedure**

0.2ml serum was added to 1ml buffer substrate, mixed and incubated for 60 minutes for AST or 30 minutes for ALT at 37°C in a water bath. Removed, added 1ml DNPH, kept at room temperature for 20 minutes, added 10ml sodium hydroxide solution, mixed and after 5 minutes read at 550nm. Blank and a series of standard were also treated in a similar manner.

Activities of AST and ALT were expressed as IU/L
Reagents: Appendix -A

Estimation of Alkaline Phosphatase (Methods of King Armstrong, 1934)

Principle

The methods used were that of King and Armstrong in which disodium phenyl phosphate is hydrolysed with liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700 nm with Folin-Ciocalteau reagent.

Procedure

Pipetted 4.0 ml the buffer substrate into a test tube and incubated at 370°C for 5 minutes. Added 0.2 ml of serum or tissue homogenate and incubated further for exact 15 minutes. Removed and immediately added 1.8 ml of diluted phenol reagent. At the same time a control was set up containing 4.0 ml buffer substrate and 0.2 ml serum to which 1.8 ml phenol reagent was added immediately. Mixed well and centrifuged. To 4.0 ml of the supernatant added 2.0 ml of sodium carbonate. Took 4.0 ml of working standard solution and for blank taken 3.2 ml water and 0.8 ml of phenol reagent. Then added 2.0 ml of sodium carbonate. Incubated all the tubes at 37 ºC for 15 minutes. Read the color developed at 700 nm.

Calculation

The activity in tissue homogenate was expressed as μ mole of phenol liberated/ min/mg protein.

Reagents: Appendix -B

Estimation of Acid Phosphatase (Methods of King Armstrong, 1934)

Principle

The enzyme catalyses the hydrolysis of phosphate esters to phosphoric acid and alcohol. The amount of phosphoric acid produced during hydrolysis is the measure of enzyme activity. The liberated phosphoric acid containing the inorganic phosphorous is estimated by Fiske and Subbarow methods.
**Procedure**

2 ml of the buffered substrate was incubated for 10 minutes at 37 °C. 0.1 ml of the enzyme was added and incubated for an hour. To the control tubes, the enzyme was added after arresting the reaction with 1 ml of 10% TCA. Centrifuged and 1.0 ml of supernatant was taken, to this 1.0 ml of molybdate solution was added followed by 0.4 ml of ANSA and made up to 10 ml with distilled water. A set of standards in the range of 10-50 μg were treated in the same manner. The colour developed was read at 660 nm after 20 minutes.

**Calculation**

The activity of acid phosphatases was expressed as micro moles of phenol liberated/minute/mg protein at 37 °C.

**Reagents: Appendix- C.**

**Estimation of Lactate Dehydrogenase (Methods of King, 1965)**

**Principle**

The lactate is acted upon by lactate dehydrogenase to from pyruvate in the presence of NAD. The pyruvate forms pyruvate phenyl hydrazone with 2, 4 dinitrophenyl hydrazine. The color developed is read in a spectrophotometer at 440 nm.

**Procedure**

Placed 1.0ml buffer substrate and 0.1 ml serum into each of two tubes. Added 0.2 ml water to the blank. Then to the test added 0.2ml of NAD. Mixed and incubated at 37 °C for 15 minutes, 1.0ml of dinitrophenyl hydrazine was added to each (test and control). Left for further 15 minutes. Then added 10ml of 0.4N sodium hydroxide and the color developed was added immediately at 440 nm. A standard curve with sodium pyruvate with the concentration range 0.1-1.0 μ moles was taken.

LDH activity in serum was expressed as μ moles of pyruvate liberated/L and in liver homogenate as μ moles of pyruvate liberated minutes/mg protein.
Reagents: Appendix -D

Estimation of Glutamate Oxaloacetate Transaminase (Reitman and Frankel, 1957)

Principle

GOT (AST) catalyses the following reaction

\[ \alpha-\text{Ketoglutarate} + \text{L – Aspарате} \leftrightarrow \text{L- Glutamate} + \text{Oxaloacetate} \]

Oxaloacetate so formed is coupled with 2,4-Dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives Brown color in Alkaline medium and this can be measured Spectrophotometrically.

Procedure

Buffered Aspartate \(\alpha\)- KG Substrate 0.25ml was taken control and Experimental samples in separate test tubes was incubated at 37 °C for 5 minutes and tissue sample 0.05mL was added mix well and incubated at 37 °C for 60 minutes and 0.25ml of DNPH color reagent was added mix well and allow to stand at room Temperature (15-30 °C) for 20 minutes and Solution I (Dilute 1 ml of reagent 3 to 10 ml with purified water) 2.5 Was mixed well and allowed to stand at Room Temperature (15-30 °C) for 10 minutes and read the O.D. against purified distilled water U.V. Spectrophotometer at 505nm.

Calculation

Mark the O.D. of Test on the Y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on X-axis.

Reagents: Appendix- E.

Estimation of Glutamate Pyruvate Transaminase

(Reitman and Frankel, 1957)

Principle

SGPT (AST) catalyses the following reaction

\[ \alpha-\text{Ketoglutarate} + \text{L – Alanine} \leftrightarrow \text{L- Glutamate} + \text{Pyruvate} \]
Oxaloacetate so formed is coupled with 2,4-Dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives Brown color in Alkaline medium and this can be measured Spectrophotometrically.

**Procedure**

Buffered Aspartate α-KG Substrate 0.25ml was taken control and Experimental samples in separate test tubes was incubated at 37 ºC for 5 minutes and tissue sample 0.05mL was added mix well and incubated at 37 ºC for 60 minutes and 0.25ml of DNPH color reagent was added mix well and allow to stand at room Temperature (15-30 ºC) for 20 minutes and Solution I (Dilute 1 ml of reagent 3 to 10 ml with purified water) 2.5 Was mix well and allow to stand at Room Temperature(15-30 ºC) for 10 minutes and read the O.D.against purified distilled water U.V. Spectrophotometer at 505nm.

**Calculation**

Mark the O.D. of Test on the Y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on X-axis.

**Reagents: Appendix F.**

**Estimation of Gamma-Glutamyl Transferase (Methods of Rosalki et al., 1970)**

**Principle**

γ-Glutamyl transferase catalyses the transfer of the γ-glutamyl group from γ-glutamyl peptides is glutathione to other peptides and to L-amino acid. γ-Glutamyl-p-nitroanilide is most commonly used as a substrate in its assay with glycylglycine as the acceptor. The enzyme catalyzes the transfer of γ-Glutamyl moiety from the substrate to glycylglycine and so liberates the chromogen-p-nitroaniline, which can be measured spectrophotometrically.

**Procedure**

Added 50 μl serum/liver homogenate, 450 μl pro-warmed buffer substrate, incubated at 37 ºC for 30 minutes, then added 2.5ml acetic acid to the reaction and read the colour at 405 nm against a blank prepared by adding 50 μl serum/liver homogenate to 450 μl buffer substrate and 2.5ml acetic acid.
**Calculation**

In an accurate spectrophotometer, the molar extinction coefficient of p-nitroaniline can be used to calculate the activity of the enzyme. $E_{405}$ of an 1 mol/l solution, 1 cm light path is 9900. So, for a solution containing 1 mmol/ml, it is 9.9.

Thus, $\gamma$-glutamyl transferase(U/L) = Reading of unknown x 202

**Reagents: Appendix- G.**

**Estimation of Bilirubin (Methods of Malloy and Evelyn Method, 1937)**

**Principle**

Bilirubin couples with diazotized sulphanilic acid to form a purple colour azobilirubin complex. The intensity of purple colour formed is proportional to the bilirubin concentration in the serum. Direct bilirubin reacts with diazo reagent in aqueous solution to form a colour dye, azo compound within 1 min. the subsequent addition of methanol accelerates the reaction of unconjugated bilirubin in the serum and value of total bilirubin is obtained after allowing the solution to stand for 30minutes. Absorbance value are taken at 540 nm.

**Procedure**

To two test tubes 0.2ml of serum and 1.8ml of distilled water were added. To one test tube 0.5ml of diazo reagent was added and considered to be the test. To the other test tube 0.5ml of 1.5% HCl was added and considered to be the blank. Then 2.5ml of methanol was added to both the tubes and allowed it to stand for 30mins. The colour developed was read at 540 nm.

For the standard curve various concentration of standard are pipette out and the volume was made upto 9 ml with methanol and 1ml of diazo reagent was added to all the tubes. The amount of directly reacting bilirubin can be determined in a similar way by substituting 2.5ml of water instead 2.5ml of methanol.
Reagents: Appendix- H

Estimation of Cholesterol (Method of Zak et al., 1953)

Principle

Cholesterol reacts with ferric chloride in the presence of concentrated sulphuric acid to give a pink color. The intensity of color developed is directly proportional to the amount of cholesterol present and is read at 540 nm in a spectrophotometer.

Procedure

To 0.1 ml of serum added 4.9 ml of ferric chloride precipitating reagent. Centrifuged and to 2.5 ml of supernatant, added 2.5 ml of ferric chloride diluting reagent. Added 4.0 ml of concentrated sulphuric acid. A blank was prepared simultaneously by taking 5.0 ml of diluting reagent. Then added 4.0 ml of concentrated sulphuric acid. After 30 minutes, the intensity of color developed was read at 540 nm against a reagent blank. The amount of cholesterol in the serum was expressed as mg/ dl and in liver as mg/g.

Reagent: Appendix I

Estimation of Liver glycogen (Grattan and Jensen, 1940)

Principle

Glycogen is treated with 45% alcohol to remove glucose. Glucose dehydrated by sulphuric acid to furfural derivative which then complexes with anthrone green colored complexes, which is read at 620 nm.

Procedure

To 0.5 ml of sample, 0.5 ml of water was added. Glucose standards were prepared by taking 0.2 -1.0ml and made upto 1.0 ml with distilled water. Added 4.0 ml of anthrone reagent to all tubes and heated in a boiling water bath for 8 min, cooled and read at 620 nm. The amount of glycogen present was expressed as mg/g tissue.
Reagents: Appendix - J

Estimation of Protein (Method of Lowry et al., 1951)

Principle

The blue color developed by the reduction of the phosphomolybdic- phosphotungstic components in the Folin- Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein the color developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured using the Lowry’s method.

Procedure

Aliquots of standard protein solution (20-100µg), 0.1ml of liver homogenate were made upto 1ml with 0.1N NaOH and shook well to treat the protein with alkali. Added 3ml of solution D, mixed well and incubated at 37ºC for 3 minutes. Added 0.3ml of solution E to each tube, mixed well and incubated further for 3 minutes at 37ºC. Read the colour developed at 750 nm against reagent blank.

Constructed a standard curve and read the concentration of protein in the aliquot taken. Calculated the concentration of protein in samples from the calibrated standard curve.

Reagent: Appendix - K

Estimation of Albumin (Method of Cooper, 1972) Dye Binding Method

Principle

The increase in absorbance of bromocresol green (BCG) at 630 nm on binding to serum albumin at pH 4.2 is directly proportional to the concentration of Albumin.

Procedure

Take 3.0 ml buffered dye solution in a series of tubes, add 10µm of serum sample vortex and read immediately (within 30 sec) the absorbance at 630 nm against blank dye solution. Analyze in duplicate and calculate the albumin content by comparing with standard run similarly.
**Reagents: Appendix –L**

**Blood Collection and Preparation of Sample for Enzymes and Molecular Analysis**

The blood sample were collected at the end of the experimental period of 60 days from the Wistar rats, that were deprived of food for overnight and the experimental animals were sacrificed by cervical dislocation and the blood was collected by puncturing the heart of the mouse and incubated in room temperature for 30 minutes. The clots were then centrifuged for 15 minutes at 2000 rpm to separate serum and used for biochemical analysis.

The liver was removed for histopathological analysis. A part of the liver was washed with ice cold tris-buffered saline, blotted dry and 10% homogenate was prepared using tris buffered saline (pH 7.4) to follow lipid per oxidation rate, 15% homogenate were prepared using phosphate buffered saline (pH 7.0) in cold condition for the estimation of antioxidants and liver marker enzymes. 20% liver homogenate were prepared using 5% trichloroacetic acid to estimate reduced glutathione. The homogenate was centrifuged at 2000 rpm for 10 minutes and the supernatant was used for the experiment.

**Micronucleus in Peripheral lymphocytes (Fenech and Morley, 1986)**

Peripheral blood samples of experimental models and control groups were collected by a heparinized sterile injector. Whole blood (0.5 ml) were added to 5 ml RPMI-1640 medium (Sigma, R0883) with 25% foetal bovine serum (Sigma, F7525), 1% L-Glutamine (Sigma, G7513), and 2% phytohaemagglutinin (Biological Industries). Cytokinesis was blocked by the method of Fenech and Morley (1986), Cytochalasin B (Serva, 18015) was added to the cultures at a final concentration of 6 g/ml 44 hour after stimulation with phytohemagglutinin. After 72 hour of incubation, cells were harvested by centrifugation, given 1 min hypotonic treatment (0.075 M KCl) and fixed in fresh fixative solution (methanol : acetic acid, 3 : 1). This fixation step was repeated twice after 20 min storage at 4°C.

In the preparation of slides, first the slides were cleaned using soap solution, washed with reverse osmosis (RO) water and kept in a beaker filled with distilled water. Before dropping cell suspension over the slide, slides were wiped with a small amount of cold fixative and the cell suspension dropped and dried over a hot plate maintained at 40°C.
The slides were stained in a horizontal staining rack, contained 2% Giesma solution made in 0.025 M phosphate buffer pH 6.8 for 5 to 8 minutes. After that, the slides were picked out and gently washed with distilled water to remove the excess stain.

In this way, the prepared slides were scanned under an oil immersion microscope (magnification 400 X) by scoring 1000 binucleated lymphocytes, in which the number of micronuclei was recorded according to standard recognition criteria (Fenech and Morley, 1986) briefly, for the scoring of micronucleus.

**The Single Cell Gel Electrophoresis assay (Comet Assay) (Singh et al., 1988)**

The Single Cell Gel Electrophoresis assay (also known as Comet Assay) is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual (Hepatocytes) Liver cells. The alkaline Comet assay was carried out according to the method described by Singh et al., 1988. It has since gained in popularity as a standard technique for evaluation of DNA damage/repair, bio-monitoring and genotoxicity testing. It involves the encapsulation of cells in a low–melting point of agarose suspension, lysis of the cells in alkaline (pH >14) conditions and electrophoresis of the suspended lysed cells. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage.

**Materials Required**

1% Normal agarose
1% Low melting agarose
Electrophoresis
Neutralization buffer
Lysis solution
Ethidium Ethydium bromide stock 1mg/ml

**Protocol**

Processing of cells
Trypsinize the cells in T20 flask and pellet it. Wash the pellet thrice in Phosphate Buffer Solution (PBS).
Electrophoresis Apparatus Set up

The apparatus used for electrophoresis is of the conventional type. The Comet assay involves the microgel electrophoresis system, gels are loaded in 18 by 18 mm area on a fully frosted microslide. Two samples, each containing about 1000-2000 cells, could be conveniently cast on each slide. 8 to 12 such slides could be placed on the platform of the electrophoresis apparatus.

Preparation of Slides

The 200µl of 1% normal agarose in PBS at 65°C on to a fully frosted micro-slide, Cover Slip and Placed over a Frozen ice pack for about 5 min and removed the cover slip after the gel had set. Mixed the cell suspension from one fraction with 1% low melting agarose at 37°C in 1:3 ratios. The 100µl of this mixture quickly on top of the gel, coated over the micro slide and allowed solidifying as before. A third coating of 100µl of 1% low melting agarose on the gel containing the cell suspension and allowed to solidified. Similarly, the slides were prepared for each cell fraction.

Cell lysis

After solidification of the agarose, the cover slips were removed and immersed the slides in ice – cold lysis solution at 4°C for 16 hours. All the above operations were performed in low lighting conditions in order to avoid any additional DNA damage.

Electrophoresis

The slides were placed horizontally in an electrophoresis tank after being removed from the lysis solution. The reservoirs filled with electrophoresis buffer until the slides just immerse in it. Allowed the slides to stand in the buffer for about 20 minutes after which carry out the electrophoresis at 0.8 v/cm for 15 minutes. After electrophoresis, remove the slides, wash thrice in neutralization buffer gently and allowed to dry. Add a few drops of the working solution of Ethidium bromide on to the gel and cover the slide with cover slip. Examine the stained DNA in the cells at 200X and 400X using a fluorescence microscope equipped with a 365 nm excitation filter and a 435 nm barrier filter. Measure the lengths of DNA migration (Comet tail) in these cells directly by fixing an ocular micrometer in one of eyepieces of the microscope. Score about 60-100 comets per point.
Statistical analysis

All the statistical analysis was performed using SPSS 13.0 for windows. Graphs were plotted using MS-Excel. The significance of variation in the Liver marker enzymes, Biochemical composition and Molecular toxic markers of Comet assay and Micronucleus assay of Alcohol Control and Control, fish oil co-administration alcohol rats Liver and Serum was analyzed by One-way ANOVA.

Results

Body Weight of the Rats

Physical changes observed in different experimental group of rat’s changes weight in different groups of rats were recorded in Table-1. Body Weight was observed that there was significance in the body weight in alcohol induction group -II (Negative Control) when compared to group I (Normal Control). The experimental group-IV (Alcohol Eel fish Oil+ Co-administration of fish oil) treated group better elevation compared to Experimental group II, III and Combined Oil group V (Plate -9; Fig. 1).

Biochemical Composition

The changes in the Protein, Cholesterol, Albumin, Bilurubin and Glycogen in the liver of control and experimental rats are illustrated in Table -2 (Plate -9 &10; Fig. 1&2). From the Table it can be revealed that there was significant increase ($P<0.05$) in experimental groups when compared to alcohol control. These levels were normalized on treatment with fish oil (90 days group- IV) Long term exposure compared with short term and Alcohol treated groups.

Assessment of liver marker Enzymes

The activity of liver marker enzymes ALT (Alkaline Transaminase), AST (Asperate Transaminase), ACP Acid Phosphatase and ALP (Alkaline Phosphatase) were assed in (Short time and long time exposed experimental animals) serum and liver homogenate in experimental and control groups. From the Table -3 (Plate - 11; Fig. 1&2) was oblivious that significant ($P <0.05$) elevation of ALT, AST, ACP and ALP in serum and liver homogenate of only alcohol administrated group (Group-II) compare to control (Group-I) and Co- administration of fish oil groups (Group III, IV and V) decreased the activity of
Table: 1 Body Weight Changes in Control and Experimental Groups rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average Weight of rats (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Day</td>
</tr>
<tr>
<td>Experiment- I</td>
<td>155.46±5.925\textsuperscript{d}</td>
</tr>
<tr>
<td>Experiment- II</td>
<td>154.20±1.090\textsuperscript{d}</td>
</tr>
<tr>
<td>Experiment- III</td>
<td>157.00±2.828\textsuperscript{d}</td>
</tr>
<tr>
<td>Experiment- IV</td>
<td>152.30±1.332\textsuperscript{d}</td>
</tr>
<tr>
<td>Experiment- V</td>
<td>156.40±1.140\textsuperscript{d}</td>
</tr>
</tbody>
</table>

Mean± SD (n=5)
Mean values within the same row sharing the same superscript are not Significant different (P>0.05)

Table: 2 Liver Biochemical levels of Control and Experimental Groups rats

<table>
<thead>
<tr>
<th>Days</th>
<th>Groups</th>
<th>Bilirubin mg/dl</th>
<th>Albumin mg/g</th>
<th>Glycogen gm/100mg</th>
<th>Protein mg/g</th>
<th>Cholesterol mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Liver</td>
<td>Liver</td>
<td>Liver</td>
<td>Liver</td>
</tr>
<tr>
<td>30 Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.75±0.084\textsuperscript{e}</td>
<td>0.88±0.108\textsuperscript{g}</td>
<td>2.38±0.894\textsuperscript{a}</td>
<td>17.69±1.439\textsuperscript{a}</td>
<td>135.19±2.303\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2.82±0.471\textsuperscript{d}</td>
<td>2.84±0.700\textsuperscript{c}</td>
<td>1.12±0.233\textsuperscript{e}</td>
<td>13.83±0.243\textsuperscript{d,e}</td>
<td>175.58±2.217\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1.86±0.114\textsuperscript{e}</td>
<td>1.93±0.095\textsuperscript{d,c,e}</td>
<td>1.35±0.029\textsuperscript{d,e}</td>
<td>14.57±0.476\textsuperscript{c,d,e}</td>
<td>144.13±2.587\textsuperscript{c}</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1.83±0.143\textsuperscript{e}</td>
<td>1.80±0.346\textsuperscript{c}</td>
<td>1.92±0.087\textsuperscript{b}</td>
<td>15.37±1.150\textsuperscript{b,c,e}</td>
<td>133.53±1.733\textsuperscript{d}</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>1.90±0.190\textsuperscript{e}</td>
<td>1.55±0.433\textsuperscript{f}</td>
<td>1.75±0.029\textsuperscript{b,c}</td>
<td>13.93±0.722\textsuperscript{d,e}</td>
<td>134.99±1.937\textsuperscript{c,d}</td>
<td></td>
</tr>
<tr>
<td>90 Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.81±0.037\textsuperscript{e}</td>
<td>3.16±0.481\textsuperscript{b}</td>
<td>1.88±0.105\textsuperscript{c}</td>
<td>17.89±1.886\textsuperscript{a}</td>
<td>137.18±2.605\textsuperscript{d}</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>6.05±0.857\textsuperscript{a,b}</td>
<td>8.49±0.481\textsuperscript{a}</td>
<td>0.64±0.031\textsuperscript{g}</td>
<td>8.79±0.679\textsuperscript{f}</td>
<td>225.86±1.743\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>3.56±0.203\textsuperscript{b,e}</td>
<td>4.50±0.470\textsuperscript{b}</td>
<td>1.30±0.346\textsuperscript{d,e}</td>
<td>13.17±1.102\textsuperscript{e}</td>
<td>153.87±3.191\textsuperscript{c}</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>2.56±0.246\textsuperscript{e}</td>
<td>3.72±0.418\textsuperscript{b}</td>
<td>1.71±0.048\textsuperscript{b,c,d}</td>
<td>16.60±0.419\textsuperscript{a,b}</td>
<td>133.96±3.050\textsuperscript{d,e}</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>2.83±0.056\textsuperscript{d}</td>
<td>6.44±0.737\textsuperscript{a,b}</td>
<td>1.06±0.031\textsuperscript{f}</td>
<td>14.85±0.206\textsuperscript{c,d}</td>
<td>135.82±0.778\textsuperscript{c,d,e}</td>
<td></td>
</tr>
</tbody>
</table>

Mean± SD (n=5)
Mean values within the same row sharing the same superscript are not Significant different (P>0.05)
Plate 9

Figure 1. Body Weight Changes in Control and Experimental Groups rats (90 Days)

Figure 2. Liver Biochemical content of Bilirubin and Albumin in Control and Experimental Groups rats (Short term and Long term Exposures)
Plate 10

Figure 1. Liver Biochemical content of Glycogen and Protein and Albumin in Control and Experimental Groups rats (Short term and Long term Exposures)

Figure 2. Liver Biochemical content of Glycogen and Protein and Albumin in Control and Experimental Groups rats (Short term and Long term Exposures)
Plate 11

Figure 1. Effect of Fish oil on Serum Marker Enzymes Aspartate Transaminase (AST) and Alanine Transaminase (ALT) Alkaline Phosphatase (ALP) and Acid Phosphatase (Short term Exposures)

Figure 2. Effect of Fish oil on Liver Marker Enzymes Aspartate Transaminase (AST) and Alanine Transaminase (ALT) Alkaline Phosphatase (ALP) and Acid Phosphatase (Long term Exposures)
Table. 3 Levels of Alanine transaminase (ALT), Aspartate transaminase (AST), Acid Phosphatase (ACP), Alkaline Phosphatase (ALP) in Liver and Serum of rats

<table>
<thead>
<tr>
<th>Days</th>
<th>Groups</th>
<th>ALT Serum: Units/ml</th>
<th>AST Serum: Units/ml</th>
<th>ACP Units: micro moles of phenol liberated/min/mg/protein</th>
<th>ALP Units: micro moles of phenol liberated/min/mg/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver: micro moles of pyruvate liberated/min/mg/protein</td>
<td>Liver: micro moles of pyruvate liberated/min/mg/protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Serum</td>
<td>Liver</td>
<td>Serum</td>
</tr>
<tr>
<td>30 Days</td>
<td>I</td>
<td>0.48±0.031&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.71±0.010&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.42±0.062&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.65±0.015&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.76±0.020&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.95±0.012&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.98±0.038&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.35±0.045&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0.67±0.020&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.08±0.012&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>0.62±0.081&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.77±0.014&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>0.67±0.017&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.19±0.012&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.59±0.074&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>0.73±0.046&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>0.61±0.014&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.02±0.120&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.48±0.045&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.69±0.014&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>90 Days</td>
<td>I</td>
<td>0.76±0.034&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.87±0.320&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.67±0.016&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.71±0.010&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>1.84±0.036&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.91±0.140&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.90±0.316&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.76±0.160&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0.87±0.022&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.87±0.075&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.73±0.033&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.23±0.012&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>0.91±0.054&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.98±0.236&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.87±0.010&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.19±0.006&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>0.79±0.058&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.23±0.102&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.98±0.014&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.15±0.506&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean± SD (n=5)

Mean values within the same row sharing the same superscript are not Significant different (P>0.05)
Plate 12

Figure 2 Effect of Fish oil on Serum and Liver Marker Enzymes Lactate Dehydrogenase (LDH), Glutamate Pyruvate Transaminase (GPT), Glutamate Oxaloacetate Transaminase (GOT) and Gamma Glutamyl Transferase (GGT) (Short term and Long term Exposures)
these serum and liver enzymes to a significant extent at $P<0.05$ in fish oil treated groups. Table-4 (Plate -12; Fig. 1&2) exhibit LDH, SGPT, SGOT and GGT activities in Serum and Liver of control and different treatment groups. A significant increase $P<0.05$ in LDH,SGPT,SGOT and GGT activity was exhibited in alcohol induced groups when compare to group-I and alcohol Co-administration with fish oil (Cod and Eel fish oil) groups showed a better reduction in group -IV, III and V rats. Apart from this enzymes long term exposed experimental groups enzymes activity comparatively better elevation compared to short term exposed groups. In the present study when short term exposure compared with Long term exposed fish oil co-administration group animals seems to be very effective.

**Results of Comet assay**

The Effect of alcohol administration and Co-administration of on the fish level of DNA damage in hepatocytes of control and experimental group was assessed by the comet assay. A total number of 5 groups were represents the Table -5. The different level of Co-administration of Cod and Spiny eel fish oil with alcohol administrated rats. The alcohol exposed groups displayed significantly higher level of DNA damage than normal group and fish oil co-administration groups. The ranges of the (MTL) mean tail length and the MTM (mean tail movement) were (22.55±8.061µm, 63.42±14.53%) and in experimental groups, respectively, while the mean tail length and mean tail movement were (12.94 ±1.306, 11.38±2.356, 19.39±2.527 and 0 ) and (2.07±2.384, 0.508±0.180 and 2.11±0.320 and 0%) in controls respectively. There was significant difference between Experimental and Control groups for 40.71±3.979 % DNA damage, MTL, and MTM ($P<0.05$) (Table- 5; Plate - 6-8; Fig. 1&2 and Plate - 13; Fig. 1).

**Results of Micronucleus**

Table- 6 (Plate -3&4; Fig.1&2; Plate -5&14; Fig.1) shows the results of the liver Micronucleus assay in Wister rats. Mean incidence (0.0437±0.02208) of Micronucleus hepatocytes in the alcohol group compare to control and fish oils administrated groups. Short term exposure of all groups’ experimental rats did not many alterations in % MNHEPs. The long term alcohol exposed rats (1.4426±0.30871%) were observed in Micronucleus frequencies were observed very high. Results of ANOVA consistently showed that there is a
Table 4 Levels of Lactate Dehydrogenase (LDH), (GPT), (GOT), Gamma-Glutamyl Transferase (GGT) in Liver and Serum of rats

<table>
<thead>
<tr>
<th>Days</th>
<th>Groups</th>
<th>LDH</th>
<th>GPT</th>
<th>GOT</th>
<th>GGT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serum: Microgm of pyruvate liberated /min /litre</td>
<td>Serum: Units/ml</td>
<td>Serum: Units/ml</td>
<td>Serum: Units:IU/litre</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver: Microgm of pyruvate liberated /min /mg protein</td>
<td>Liver: micro moles of Pyruvate liberated /min/mg/protein</td>
<td>Liver: micro moles of pyruvate liberated /min/mg/protein</td>
<td>Liver: Serum</td>
</tr>
<tr>
<td>30 Days</td>
<td>I</td>
<td>36.32±0.816&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.76±2.440&lt;sup&gt;f&lt;/sup&gt;</td>
<td>65.33±0.693&lt;sup&gt;f&lt;/sup&gt;</td>
<td>11.21±1.474&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>67.32±4.242&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.76±0.778&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68.70±2.097&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23.07±1.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>42.76±3.559&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.13±4.082&lt;sup&gt;d&lt;/sup&gt;</td>
<td>63.72±0.920&lt;sup&gt;g&lt;/sup&gt;</td>
<td>12.25±0.79&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>43.98±1.632&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.54±3.162&lt;sup&gt;e&lt;/sup&gt;</td>
<td>68.96±0.206&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17.27±0.426&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>40.94±6.531&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.61±2.82&lt;sup&gt;e&lt;/sup&gt;</td>
<td>62.44±0.417&lt;sup&gt;h&lt;/sup&gt;</td>
<td>18.93±1.021&lt;sup&gt;l&lt;/sup&gt;</td>
</tr>
<tr>
<td>90 Days</td>
<td>I</td>
<td>37.54±2.449&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>24.65±5.656&lt;sup&gt;c&lt;/sup&gt;</td>
<td>67.40±0.469&lt;sup&gt;h&lt;/sup&gt;</td>
<td>11.77±0.272&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>87.49±2.943&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.88±3.265&lt;sup&gt;d&lt;/sup&gt;</td>
<td>178.66±0.833&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.52±0.994&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>43.08±3.559&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.26±1.290&lt;sup&gt;d&lt;/sup&gt;</td>
<td>72.91±0.060&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.05±0.659&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>44.65±2.828&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.87±1.414&lt;sup&gt;e&lt;/sup&gt;</td>
<td>70.85±0.645&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.0±0.816&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>42.78±2.160&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.91±1.632&lt;sup&gt;de&lt;/sup&gt;</td>
<td>60.46±0.646&lt;sup&gt;i&lt;/sup&gt;</td>
<td>26.67±0.483&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean± SD (n=5)
Mean values within the same row sharing the same superscript are not Significant different (P>0.05)
Table 5. Covariable effect of Spiny Eel and Cod Liver oil on AlcoholExposed rat Comet assay in Hepatocytes

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Dose (ml/kg)</th>
<th>No. of rats</th>
<th>No. of Mortality</th>
<th>Exposure Periods (Days)</th>
<th>No. of Cells Analyzed</th>
<th>% of DNA Damage</th>
<th>Tail Length (µm)</th>
<th>Mean Tail Movement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control (+ “ve”)</td>
<td>Standard Diet</td>
<td>6</td>
<td>-</td>
<td>90</td>
<td>100</td>
<td>0c</td>
<td>0d</td>
<td>0c</td>
</tr>
<tr>
<td>Negative Control (-“ve”)</td>
<td>Standard Diet +3ml of 40% Alcohol Thrice a Day Orally Administrated</td>
<td>6</td>
<td>2</td>
<td>90</td>
<td>100</td>
<td>40.71±3.979a *(0.39716)</td>
<td>63.42±14.536a *(01.45126)</td>
<td>22.55±8.061a *(0.70544)</td>
</tr>
<tr>
<td>Ex1 (Cod Liver Oil)</td>
<td>Standard Diet +3ml of 40% Alcohol+ Cod Liver Oil(5ml/kg) Thrice a Day Orally Administrated</td>
<td>6</td>
<td>-</td>
<td>90</td>
<td>100</td>
<td>12.26±1.306b *(0.13356)</td>
<td>12.94±2.734c *(0.27190)</td>
<td>2.07±2.384b *(0.25512)</td>
</tr>
<tr>
<td>Ex2(Spiny Eel Oil)</td>
<td>Standard Diet +3ml of 40% Alcohol+ Spiny Eel Oil(5ml/kg) Thrice a Day Orally Administrated</td>
<td>6</td>
<td>-</td>
<td>90</td>
<td>100</td>
<td>4.42±0.776d *(0.08011)</td>
<td>11.38±2.356c *(0.23753)</td>
<td>0.508±0.180c *(0.01812)</td>
</tr>
<tr>
<td>Ex3 (Combined Fish oil-Cod+Eel)</td>
<td>Standard Diet +3ml of 40% Alcohol+ Combined Oil (Cod and Eel oil (5ml/kg) Thrice a Day Orally Administrated</td>
<td>6</td>
<td>-</td>
<td>90</td>
<td>100</td>
<td>10.95±1.191c *(0.11973)</td>
<td>19.39±2.527b *(0.25496)</td>
<td>2.11±0.320b *(0.03299)</td>
</tr>
</tbody>
</table>

Mean± SD (n=5)
SE (n=5)
Mean values within the same row sharing the same superscript are not Significant different (P>0.05)
Plate 6. Florescent Microphotographs of Comet image of Control and Experiment Group rat Hepatocytic Cells

Figure 1. Normal Control group rats Liver Hepatocytes Cell Comet

Figure 2. Rats treated with Alcohol groups Liver Hepatocytic Cells Comet
Plate 7

Figure 1. Rats treated with Alcohol and Co-administrated of cod fish Oil Liver Hepatocytic Cells Comet

Figure 2. Rats treated with Alcohol and Co-administrated of Spiny eel fish Oil Liver Hepatocytic Cells Comet
Plate 8

Figure 3. Rats treated with Alcohol and Co-administrated of Combined fish Oil (Cod and Eel fish Oil) Liver Hepatocytic Cells Comet
Plate 13

Figure 1. Effect of Alcohol on the induction of DNA damages in rats Hepatocytic cells and Co administrations of fish oils with alcohol groups % DNA Damage, Comet Tail Length and Mean Tail Movement
<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Dose (ml/kg)</th>
<th>No. of rats</th>
<th>No. of Mortality</th>
<th>Exposure Periods (Days)</th>
<th>No of Cells Analyzed</th>
<th>MNHEP%/2000 Mean Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control (+ve)</td>
<td>Standard Diet</td>
<td>6</td>
<td>-</td>
<td>90</td>
<td>2000</td>
<td>0.0437±0.02208 *(0.00049)</td>
</tr>
<tr>
<td>Negative Control (-ve)</td>
<td>Standard Diet +3ml of 40% Alcohol Thrice a Day Orally Administered</td>
<td>6</td>
<td>2</td>
<td>90</td>
<td>2000</td>
<td>1.4226±0.30871 *(0.00690)</td>
</tr>
<tr>
<td>Ex1 (Cod Liver Oil)</td>
<td>Standard Diet +3ml of 40% Alcohol+ Cod Liver Oil(5ml/kg) Thrice a Day Orally Administered</td>
<td>6</td>
<td>-</td>
<td>90</td>
<td>2000</td>
<td>0.4612±0.40606 *(0.00896)</td>
</tr>
<tr>
<td>Ex2 (Spiny Eel Oil)</td>
<td>Standard Diet +3ml of 40% Alcohol+ Spiny Eel Oil (5ml/kg) Thrice a Day Orally Administered</td>
<td>6</td>
<td>-</td>
<td>90</td>
<td>2000</td>
<td>0.2493±0.25600 *(0.00572)</td>
</tr>
<tr>
<td>Ex3 (Combined fish oil (Cod+Eel Oil))</td>
<td>Standard Diet +3ml of 40% Alcohol+ Combined Oil (Cod and Eel oil (5ml/kg) Thrice a Day Orally Administered</td>
<td>6</td>
<td>-</td>
<td>90</td>
<td>2000</td>
<td>0.3616±0.04633 *(0.00104)</td>
</tr>
</tbody>
</table>

Mean values within the same row sharing the same superscript are not significantly different (P>0.05).
Plate 3. Photograph of Micronuclei in the Control and Experimental Groups rats

Figure 1. Normal Control group rats Micronuclei

Figure 2. Alcohol (Negative control) groups rats Hepatocytic Micronuclei
Plate 4

Figure 1. Alcohol Co administrated of Cod fish oil treated groups rats Hepatocytic Micronuclei

Figure 2. Alcohol Co administrated of Eel fish oil treated groups rats Hepatocytic Micronuclei
Plate 5

Figure 1. Alcohol Co administrated of combined fish oil treated groups rats
Hepatocytic Micronuclei
Plate 14

Figure 1 Micronucleus cell induced by Alcohol and Co administrations of fish oil Rat groups
significant difference ($P<0.05$) in mean numbers of MNHEPs among the fish oil co-administrated groups. Results of the Analysis of Variance showed that only alcohol exposure was significantly related with the number of micronucleus depended on durations of exposures and treated with fish oil co administrations of rats. The present study were proved some declines of micronucleus spiny eel oil co-administrated rat hepatocytes and the results of the study suggest that fish oil have a strong genotoxic effect of the alcohol induced liver damage and also conformations of liver marker enzymes.

**Discussion**

The present study body weight were analyzed alcohol treated experimental groups and normal control group short term exposure not much alteration rats in normal liver (30 days). Toxic effects of alcohol long term (90 days) exposed group weight negatively correlated between time duration and co -administration of fish oil group regeneration of weight and Liver enzymes compare to alcohol administration groups. In previous studies, when rats were given ethanol as an isocaloric substitution for carbohydrates, a lower rate of weight gain could be seen (Reinus *et al.*, 1989). Das *et al.*, 1995 reported that reduction due to alcohol ingestion is due to the toxic effect of ethanol. These observations are also in perfect conformity with Venkumar *et al.*, (2000). The body weight Significant differences between Experimental and control ($P>0.05$). However, the total energy intake in the ethanol-exposed groups was due to the ethanol. Ethanol probably alters the metabolic rate, which may explain its failure to promote weight gain (Leibel *et al.*, 1995).

Higher levels of AST and ALT in the circulation indicated disintegration of cell membrane of liver. Prolonged destruction in the hepatic cells results in more cytoplasmic releases to exacerbate hepatic dysfunction and causes an elevation of ALP, LDH and bilirubin in serum (Monika and Nirala, 2009). The damage provoked by free radicals to macromolecule plays an essential role in the pathophysiological process of atherosclerosis, Inflammation, carcinogenesis, aging, drug reaction and toxicity. Alcohol-induced hepatic tissue damage is mediated by acetaldehyde and reactive oxygen species Zima *et al.*, 2001. The removal and neutralization of these noxious toxic metabolites are considered to be vital initial steps in the prevention of alcohol-related liver diseases (Faemi *et al.*, 2008). In the present study, fish oils decreased the levels of AST and ALT towards the respective control values that were an indication of stabilization of plasma membrane as
well as repair of hepatic tissue damage caused by alcohol. Usually, the extent of hepatic damage is assessed by the increased level of cytoplasmic enzymes (ALT, AST and ALP), thus leads to leakage of large quantities of enzymes into the blood circulation. This was associated by massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver (Shankar et al., 2008). When liver cell plasma membrane is damaged variety of enzymes normally located cytosol are released. The estimation of these enzymes is a useful quantitative marker of the extent and type of hepatocellular damage (Venukumar et al., 2002). Cellular damage exhibits good correlation with the enzyme leakage (Sherawat and Sultana, 2006). Serum AST, ALT, ALP, γ-GT and bilirubin are the most sensitive markers employed in the diagnosis of hepatic damage (Sallie et al., 1991). The excess consumption of alcohol has been well associated with distorted damage and metabolism in liver along with leakage of cytoplasmic liver enzymes into the blood (James et al., 1993). AST and ALT are considered among the most sensitive markers of hepatocellular injury. ALP, which is secreted from the lysosomes, is also a marker enzyme for assessing liver damage (Singha et al., 2007). When the integrity of the lysosomal membrane changes and/or the membrane of the lysosome are ruptured by deleterious influences, this acid hydrolase enters the blood stream, producing transient increase in the activity of lysosomal enzymes in the serum. The increased levels of these enzymes (AST, ALT and ALP) in the serum have been observed in alcohol administered rats, which indicate increased permeability, damage and necrosis of hepatocytes (Adewusi and Afolayan, 2010). Significantly decreased levels of serum enzyme markers, thus suggesting that the extract possessed compounds that protected. Elevated generation of reactive oxygen species (ROS) or free radicals causes auto-oxidation of hepatic cells, resulting in marked hepatic lesions (Suzuki et al., 1998) therefore indicating leakage of cellular enzymes in to the serum (Baldi et al., 1993). Kumar Rajagopal et al., 2003, demonstrated that serum levels of AST or ALT increased in ethanol administrated rats by damage of both hepatic cellular and mitochondrial membranes. The increased activities of liver marker enzymes such as ALT, AST, ALP and GGT have previously been detected in alcohol administrated mice (Pari and Suresh, 2008) implying the increased permeability, damage and necrosis of hepatocytes. In the present study, decreased activities of tissue ACPase and ALPase might be due to the membrane fragility and
permeability of the organs, whereas activity of ACPase was increased due to lysosomal activation. Propolis prevented oxidative membrane damage (Bhadauria et al., 2007) and could help in the absorption and utilization of various minerals due to the presence of organic acid derivatives in it (Haro et al., 2000), which in turn improved the physiological functions by regulating ion dependent enzymatic activities.

Alcohol significantly decreased the activities of ALPase, ACPase and GGTase in the liver homogenate. The decrease in membrane enzyme activities might have occurred due to loss of membrane enzyme and other components indicating adverse effects of alcohol on membrane integrity. The present results show that in contrast to alcohol treatment, dietary supplementation of Fish oil to control rats caused significant increase in the activities of membrane enzymes in the liver homogenate. Prior to or along with alcohol treatment, Fish oil supplementation prevented/retarded alcohol induced decrease of membrane enzymes in the tissue. The activity of lysosomal enzyme, ACPase was significantly increased in liver homogenate by alcohol treatment. Alteration in ACPase activity demonstrates cisplatin induced loss of lysosomal function (Kuhlmann et al., 1997; Courjault-Gautier et al., 1995). However, the alcohol induced effect on lysosomal enzyme activity appeared to be ameliorated at least to some extent by dietary Fish oil supplementation. Elevated levels of serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic Transaminase (SGPT) are indications of hepatocellular injury (Yue et al., 2006). In the present study Cod liver and Spiny eel oil at a dose of 5ml/kg, caused a significant inhibition in the levels of SGOT and SGPT towards the respective normal range and this is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by ethanol. The confirmation of present work SGPT, SGOT and ALP levels alteration CCl₄, Paracetamol and ethanol induced rats (Firdous et al., 2011).

The present study indicates that experimental animals exposed to alcohol, in the particular conditions of exposure of this collective evaluated, show clear evidence of Genotoxicity activity in their hepatocytes. Alcohol drinkers with ALDH₂-deficient genotype are known to be able to accumulate acetaldehyde in the body easier than some other genotypes and, consequently, to suffer from its genotoxic damage (Singh and Khan, 2010). From our results it seems that the comet assay can be considered a very promising tool in
biomonitoring studies on occupationally exposed rats, no significant difference was found in smokers to non-smokers (Morretti et al., 2000), Pesticides (Undegur and Basaran, 2002 and Carbonell et al., 1995), Alcoholics have been found to have a higher frequency of chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and micronuclei (MN), in their lymphocytes than non-alcoholics (Maffei et al., 2000), which is in line with the results of our study, with some slight differences. Since micronucleus analysis represents a cytogenetic end-point which can detect mutagenic effects due to both chromosome breakage and loss, therefore it is important to study the clastogenic and spindle poisoning agent for micronuclei induction (Chaudhary et al., 2006). The Micronucleous observed in the peripheral blood lymphocytes reflect a complex exposure environment, including bone marrow, blood stream, cell cultures, or mutagen challenge in vitro. Similar result was observed in toxicity exposures (Norppa, 1997; Iarmarcovai et al., 2008).

DNA damage was detected in liver cells using the comet assay following in vivo administration of alcohol in rats. The results are in agreement with recent work which showed an increase in the incidence on micronucleated hepatocytes following a single dose of 2,6-DNT as low as 50mg/kg or repeated dose previous dose as low as 33 mg/kg-day (Takasawa et al., 2010). When doing the comet assay on different tissues, it has to be noted that the concentrations of alkali labile sites can vary in DNA from different tissues and animal species. Some of the factors that can influence DNA strand damage in a particular tissue include cell type heterogeneity, cell cycle, cell turnover frequency and culture or growth conditions. Different cell types may have very different background levels of DNA single-stand breaks due to variation in excision repair activity, metabolic activity, anti-oxidant concentrations or other factors (Tice, 1995).

Omega PUFAs present in the eel oil inhibit the arachidonic acid pathway. Eicosapentanic acid (EPA) and Docosahexanic acid (DHA) which are abundant in fish oil, suppress colon carcinogenesis in experimental animals (Takahashi et al., 1997). Conjugated linoleic acids are reported to show anticarcinogenic properties (Narisawa et al., 1991). Meanwhile, PUFAs are like to be good target for free radical attack. Lipid peroxidation appears to be an important source for the formation of exo cyclic propane, etheno and malondialdehyde in DNA (Ha et al., 1990).
The study demonstrates the fish oil, enhancing ethanol membrane remodeling by a physical and/or oxidative process, and it can increase dramatically phosphatidyl inositol phospholipase C translocation into lipid rafts, secondarily leading to the accumulation of lysosomes and elevation in low molecular weight iron content. This rise in low molecular weight iron by eicosapentaenoic acid (EPA) can in turn be considered the key component underlying the final increase in ethanol induced oxidative stress and cell death. Even if n-3 PUFAs via membrane remodeling have been described to be beneficial in several diseases (Yaqoob, 2009), our findings suggest remaining cautious with supplements because of the possibility of simultaneous lipid Raft-dependent production of Reactive Oxygen Species (ROS). These results support the notion that *M.armatus* fish oil may be effective dietary supplements in the management of various diseases in which oxidant/antioxidant defence mechanisms are decelerated. Further, human studies are required especially in various disease states to assess both the underlying antioxidant protective mechanisms of fish oil supplementation and potential role of oxidative stress in certain diseases. This study confirms that co-administration of Spiny eel oil and Cod fish oil with ethanol attenuates the increase in lipid peroxidative damage, restores antioxidant status, markers of hepatic injury in these animals. These findings show that *M. armatus* (fish oil) is a promising candidate for chemoprevention of ethanol-induced Hepatic dysfunction. However, further detailed studies are required to establish the toxicity and protective effect of this fish oil on ethanol-induced liver disorders before it can be recommended for clinical trials.