SUMMARY AND CONCLUSION

ANTIATHEROGENIC STUDY

The male albino (Sprague Dawley) rats used for the 90 days feeding study were made atherogenic by feeding atherogenic diet (AD). The test groups fed AD were supplemented with 1% cuttlefish liver oil (CFLO), antioxidants (vitamin E & C, green tea flavonoid) both in combination and alone. The animals fed AD showed an increased gain in body weight and liver weight. Treatment with CFLO significantly decreased the body weight and this was found to be lowered further on supplementation with antioxidants to the AD + CFLO diet. However, the gain in body weight and liver weight was found to be significantly lowest in the groups fed AD + CFLO supplemented with flavonoids when compared to rats fed AD alone which could be due to the synergistic action of CFLO and flavonoid. The decrease in weight gain in CFLO treated rats could be attributed to the ω-3 fatty acids of the CFLO. The weight reduction and decrease in serum LDL–cholesterol (LDL-C) are important for lowering the risk of cardiovascular diseases (CVD).

The total cholesterol, triglycerides and phospholipids in serum and tissues were found to be increased significantly in the atherogenic rats. The LDL-C was found to be increased and the HDL-C was found to be decreased. The CFLO supplementation significantly reduced cholesterol, triglycerides, phospholipids, LDL-C and increased the HDL-C in serum. The above finding could be due to enhanced hepatic fatty acid β-oxidation since ω-3 fatty acids increase the rate of oxidative metabolism of fats. The hypolipidemic effect of CFLO was found to be further increased by supplementation with antioxidants (vitamin E & C, flavonoids). This proves the synergistic action of vitamin E & C and also the hypocholesterolemic action of the green tea polyphenols. The lipid components in rats fed AD + antioxidants alone were found to be
significantly lower than those in rats fed AD, but higher than those in rats fed AD + CFLO. This clearly indicates that the lipid lowering action of CFLO can be further enhanced by supplementation with antioxidants. The increased HDL level observed in the CFLO supplemented rats would have inhibited the LDL uptake by the arterial wall and at the same time facilitated the transport of cholesterol from peripheral tissues to the liver where it could be catabolised or discarded from the body.

The inclusion of CFLO has also produced a lowering of the atherogenic ratio compared to the AD fed rats. The effect of lowering atherogenic ratio was significantly more pronounced in rats fed CFLO + flavonoid / vitamin E&C, which indicates that supplementation of unsaturated fatty acids in the therapy of CVD, should include the co-administration of antioxidants in order to prevent fatty acid decomposition in the case of oxidative insult. The decrease in the ratio decreases the risk for the occurrence of atherosclerosis.

Increased faecal excretion of bile acids and neutral sterols has also been observed in the rats fed AD + CFLO. This was found to be further increased by incorporation of vitamin E & C or green tea flavonoids to the AD + CFLO diet, the effect being more in flavonoid supplementation. This indicates the synergistic effect of ω-3 and flavonoids. Increased bile acid excretion would reduce cholesterol concentrations because the plasma or liver cholesterol would be utilized to maintain the bile acid pool.

The biosynthetic enzyme HMG CoA reductase was found to be decreased in the atherogenic rats. The glycolytic enzymes like glucose-6-phosphate dehydrogenase and malic enzyme also showed a decreased activity in atherogenic rats. The inclusion of vitamin E & C to the AD fed rats showed no significant difference in the activities of these enzymes when compared to the AD group. The addition of CFLO or green tea flavonoids to the AD brought
about further decrease in the enzyme activities. A combined supplementation of AD with CFLO + flavonoids brought about the highest inhibition of the lipogenic enzyme activities. The effect may be due to the presence of ω-3 PUFAs in CFLO which are known to inhibit lipogenesis and stimulate fatty acid β–oxidation. Green tea flavonoids are also known to inhibit lipogenesis and promote fatty acid oxidation.

The antioxidant enzymes and antioxidant content were found to be significantly lowered in the rats fed AD. There was also an increase in lipid peroxidation products like MDA and CD which confirmed the condition of atherosclerosis as lipid peroxidation potentially contribute to the increased incidence of atherosclerosis. Supplementation of AD with CFLO and/or antioxidants significantly increased the antioxidant enzyme activities and concentration of antioxidants, but decreased the concentration of peroxidation products. These findings can be due to the action of ω-3 PUFAs as inhibitors of free radical generation, the protective action of vitamin E on cells by inhibiting the peroxidation of PUFA in membrane phospholipids and due to the protective action of flavonoid on circulating and membrane lipids by sparing vitamin E and endogenous antioxidants. Hence, the supplementation of AD with CFLO + antioxidants was found to be more effective in boosting the antioxidant defense system, proving the protective role of the antioxidants on PUFA in cellular membranes and on lipoproteins from oxidation into hydroperoxides. Therefore, it can be inferred that in the presence of antioxidants the CFLO was available to inhibit free radical generation and thus stimulate the antioxidant defense system.

The study also shows that the supplementation of antioxidants to the AD + CFLO diet increased the beneficial aspects of the ω-3 fatty acid content of CFLO by sparing it for lowering the blood lipid components and inhibition of lipogenesis & free radical generation.
The above findings establish the antiatherogenic activity of CFLO at 1% level in the diet. The high content of ω-3 fatty acid of CFLO can be ascribed to be the factor responsible for the antiatherogenic activity. Supplementation of CFLO with vitamins E & C or green tea flavonoids further enhanced the antiatherogenic activity, the most effective combination being CFLO + flavonoid supplement. The antiatherogenic action observed is through decreased lipogenesis, increased cholesterol transport to liver, enhanced excretion of neutral sterols and bile acids and above all a stimulated antioxidant defense system.

**CARDIOPROTECTIVE EFFECT**

The animals were grouped as 1a & 1b- fed on normal diet; Groups-2a & 2b which were fed on normal diet + 1% cuttlefish liver oil, for a period of 45 days. Myocardial infarction was induced in animals of 1b (fed on normal diet) and in 2b (fed on normal diet + 1% CFLO) by subcutaneous injection of isoproterenol [6mg (dissolved in physiological saline) per 100g body weight] twice at an interval of 24h at the end of 45 days. Simultaneously, the control animals (1a and 2a) were injected with physiological saline alone.

The administration of isoproterenol to Group-1b rats resulted in the induction of myocardial infarction as was evident from the increased levels of marker enzymes namely LDH, CPK, GPT, GOT and CK-MB. The prior administration of 1% CFLO along with feed to the Group-2b animals decreased the activities of these marker enzymes as compared to group 1b isoproterenol-injected rats.

The lower activities of the marker enzymes in the 1% CFLO treated group (2b) compared to that of control group (1b), highlights the cardioprotective effect of the ω-3 fatty acids, EPA and DHA present in CFLO, since these PUFAs being highly unsaturated get incorporated in the membrane phospholipids and offer protection against tissue damage.
ANTI-INFLAMMATORY AND PLATELET AGGREGATION INHIBITING ACTIVITIES

At the end of 45 days feeding study, the anti-inflammatory activity was determined by carrageenan induced acute and formalin induced chronic paw edema models in the control animals (fed on normal diet) and test animals (fed on normal diet + 1 % CFLO).

The beneficial effects observed can be attributed to the ω-3 PUFAs present in CFLO, which most likely relate to their modification of eicosanoid synthesis and metabolism. The treatment with CFLO to the test animals was found to produce significant anti-inflammatory activity in acute and chronic inflammations compared to the control animals.

Addition of ADP to platelets separated from the blood of control animals was found to show aggregation of platelets, whereas, in the case of platelets isolated from the test animals the ADP induced platelet aggregation was found to be inhibited. The above findings could be due to the EPA content of the CFLO, which can serve as a precursor of TXA₃ and PGI₃, where the TXA₃ does not induce aggregation of platelets and PGI₃ like PGI₂ is a potent antiaggregating agent. EPA may also affect platelet aggregation by blocking TXA₂ receptors on the cell membrane or by the formation of prostaglandins D₃ and E₃ as opposed to prostaglandins D₂ and E₂.

The above findings reveal the beneficial effects of feeding 1% CFLO on suppression of inflammatory response and inhibition of platelet aggregation in rats which can be said to be due to the high EPA content of the CFLO.

IMMUNOSTIMULATORY ACTION

The immunostimulatory action was determined by assaying the Splenic T-lymphocyte mitogen response, bone marrow cell proliferation assay, plaque formation cell assay and
circulating antibody titre, at the end of 45 days feeding of the control rats with normal diet and the test animals with normal diet + 1% CFLO.

In the cuttlefish liver oil treated animals, the spleen cell proliferation was found to be stimulated in the presence of mitogen as seen from the increased \(^{3}\text{H}\) Thymidine incorporation. Enhanced proliferation of bone marrow cells was also observed in treated animals compared to control animals. These findings indicate induction of proliferation of bone marrow stem cells either directly or indirectly, stimulating the release of factors that are involved in the regulation of hemopoiesis. The treated animals also showed an increase in number of plaque forming cells in the spleen and antibody titre in the circulation, which are the functions of B-cells.

The results show that the EPA rich CFLO boosted the immune function, which could have been primarily by mediating the eicosanoid production by decreasing those that are pro-inflammatory and increasing those that are anti-inflammatory. The above results indicate that feeding CFLO at a level of 1% in the diet for 45 days stimulated the immune function and inhibited the inflammatory response in the experimental animals.

The CFLO extracted from the liver of *Sepia pharaonis* Ehrenberg, was hence found to possess very good antiatherogenic, cardioprotective, anti-inflammatory, platelet aggregation inhibiting and immunostimulating properties, as established by the biochemical studies. The high content of \(\omega-3\) PUFAs (EPA and DHA) present in the CFLO can be ascribed to be the factor responsible for the above beneficial effects.