CHAPTER 5
ANTI-INFLAMMATORY AND PLATELET AGGREGATION INHIBITING ACTIVITIES OF CUTTLEFISH LIVER OIL

5.1 INTRODUCTION

A range of anti-inflammatory effects of the ω-3 family of polyunsaturated fatty acids, particularly those found in fish oils have been identified. It has been ascertained that these fatty acids work in part by antagonizing the production and action of arachidonic acid (AA)-derived eicosanoids and in part by eicosanoid independent mechanisms (Calder, 2001). The ω-3 PUFAs when available in diet, produce a series of eicosanoids (20 C metabolites- prostanoids and leukotrienes) which displace and/or modify the effects of those synthesized from ω-6 PUFAs (Pigott and Tucker, 1990). The beneficial effects of ω-3 PUFAs most likely relate to their modification of eicosanoid synthesis and metabolism. Arita et al. (2005) reports that the anti-inflammatory effect of fish oils appears to be due to a powerful anti-inflammatory compound called resolvin (resolution-phase interaction product) E1, which is produced from eicosapentaenoic acid (EPA). At nanomolar levels, resolvin E1 was found to reduce dermal inflammation, peritonitis, dendritic cell migration, and interleukin (IL)-12 production. In this chapter we report the anti-inflammatory and platelet aggregation inhibiting activities in male albino Sprague Dawley rats fed 1% cuttlefish liver oil in diet.

5.2 MATERIALS AND METHODS

5.2.1 ANIMALS

Male albino Sprague Dawley rats weighing 80-110 g body weight, purchased from Small Animal Breeding Centre, Kerala Agricultural University (KAU), Thrissur, were used for the study.
5.2.2 **PREPARATION OF CUTTLEFISH LIVER OIL**

The cuttlefish liver oil was prepared as described in section 2.2.1

5.2.3 **EXPERIMENTAL DESIGN**

The animals were housed in groups of six in polypropylene cages with a 12:12 light/dark cycle. Sufficient number of control groups and test groups were maintained so that at least 6 animals were available for each assay. The animals in the control group were fed on normal diet purchased from College of Veterinary and Animal Sciences, KAU, Thrissur. The animals of the test group were fed on normal diet + 1% cuttlefish liver oil. They were provided with food and water *ad libitum*. The experimental duration was 45 days.

5.2.4. **ANTI-INFLAMMATORY ACTIVITY**

The anti-inflammatory activity was determined by carrageenan induced acute and formalin induced chronic paw edema models.

5.2.4.1 **CARRAGEENAN INDUCED PAW EDEMA**

Carrageenan induced paw edema was used for determining the acute anti-inflammatory activity of the liver oil. In both the control and test groups, inflammation was produced by injecting 0.1 ml of a 1% carrageenan solution in the left hind paw of the rat. The paw thickness was measured using vernier calipers before and 3 h after carrageenan injection (Winter *et al.*, 1962).

Increase in paw thickness was calculated using the formula $P_t - P_o$, where $P_t$ is the thickness of paw at time ‘t’ (i.e. 3h after carrageenan injection) and $P_o$ is the paw thickness at ‘0’ time. Percentage edema was calculated using the formula $\left(\frac{(P_t - P_o)}{P_t}\right) \times 100$. 

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5.2.4.2 FORMALIN INDUCED PAW EDEMA

Chronic anti-inflammatory activity was determined by formalin induced paw edema (Chau, 1989). In both the control and test groups, chronic inflammation was produced by injecting 0.1 ml of 2 % formalin in the left hind paw of rat. The paw thickness was measured using vernier calipers before and 6 days after formalin injection.

Increase in paw thickness was calculated using the formula $P_t - P_o$, where $P_t$ is the thickness of paw at time ‘t’ (i.e. 6 days after formalin injection) and $P_o$ is the paw thickness at ‘0’ time. Percentage edema was calculated using the formula $[(P_t - P_o) / P_t] \times 100$.

5.2.4.3 PLATELET AGGREGATION INHIBITING ACTIVITY

For determining the platelet aggregation inhibiting activity, the blood from control animals and test animals was collected in anticoagulant solution (2.4% sodium citrate, 1.5% citric acid and 1.8% dextrose). The ratio of blood to anticoagulant solution was approximately 5:1. The platelet rich plasma (PRP) was separated by centrifugation at 1850 rpm for 7 min. PRP was centrifuged at 4530 rpm for 18 min. to sediment the platelets (Chopra et al., 1999). The platelet sediment was dispersed in washing buffer, which was composed of 113 mM NaCl, 4.3 mM KH$_2$PO$_4$, 4.3 mM Na$_2$HPO$_4$, 24.44 mM NaH$_2$PO$_4$ and 5.5 mM dextrose, pH 6.5 and the platelets were collected after centrifugation at 900 x g for 10 min. Then platelets were suspended in a buffer composed of 109 mM NaCl, 4.3 mM K$_2$HPO$_4$, 16 mM Na$_2$HPO$_4$, 8.3 mM NaH$_2$PO$_4$ and 5.5 mM dextrose, pH 7.5 (Baeziger and Majerus, 1974). The suspension was adjusted to give a final optical density of approximately 0.5 at 600 nm. To one ml of platelet suspension 20 μl of 1mM ADP was added and the OD at 600 nm was measured at 1 min. intervals upto 5 min. in a Spectrophotometer (Jasco V-530, Japan).
5.3 RESULTS

5.3.1 CARRAGEENAN INDUCED PAW EDEMA

The mean % edema after 3 h of carrageenan injection was significantly lower in the test animals (Table-5.1).

5.3.2 FORMALIN INDUCED PAW EDEMA

Formalin induced paw edema was inhibited significantly in the animals fed on cuttlefish liver oil. (Table-5.1).

5.3.3 PLATELET AGGREGATION INHIBITING ACTIVITY

Addition of ADP to platelets separated from the blood of control animals showed a decrease in OD at 600 nm indicating aggregation of platelets (Fig-5). But in the case of platelets isolated from the test animals the decrease in OD at 1 minute after adding ADP was half that of the control and the OD slowly increased showing that ADP induced platelet aggregation was inhibited in the cuttlefish liver oil fed animals.
Table 5.1 Effect of cuttlefish liver oil on carrageenan induced acute inflammation and formalin-induced chronic inflammation in rats.

<table>
<thead>
<tr>
<th>Model</th>
<th>Carrageenan model</th>
<th>Formalin model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Mean % edema after 3 h</td>
<td>Mean % edema after 6 days</td>
</tr>
<tr>
<td>Control</td>
<td>140.52 ± 2.64</td>
<td>250.23 ± 2.4</td>
</tr>
<tr>
<td>Test</td>
<td>60.60 ± 1.45*</td>
<td>110.34 ± 1.7*</td>
</tr>
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*P < 0.001; Values are mean ± SD of six different estimations.

Fig.5 Platelet aggregation inhibiting activity of cuttlefish liver oil
In the present study the CFLO exhibited significant anti-inflammatory activity in acute and chronic inflammations in rats. Carrageenan induced acute inflammation in animals is one of the most suitable test procedures to screen anti-inflammatory agents. The development of carrageenan induced edema is biphasic, the first phase is attributed to the release of histamine, 5-HT and kinins and occurs within an hour of injection and is partly due to the trauma of injection, while, the second phase is related mainly to prostaglandins (PGs) which is measured around 3h (Larsen and Henson, 1983; Vane and Booting, 1987). The formalin induced paw edema is one of the most suitable test procedures to screen chronic anti-inflammatory agents, as it closely resembles human arthritis (Greenwald, 1991). The nociceptive effect of formalin is also biphasic, an early neurogenic component followed by a later tissue mediated response (Wheeler- Aceto and Cowan, 1991). The ability of fish oil to reduce acute and chronic inflammatory response has been well established. The ω-3 PUFAs may exert their effects by modulating signal transduction/or gene expression within inflammatory and immune cells (Calder, 1998). EPA is a substrate for cyclooxygenase and lipooxygenase and gives rise to mediators that often have different biological actions or potencies than those formed from arachidonic acid. Clinical studies have reported that fish oil supplementation has beneficial effects in rheumatoid arthritis, inflammatory bowel disease and some asthmatics, supporting the idea that the ω-3 PUFAs in fish oil are anti-inflammatory (Calder, 2001).

Arita et al. (2005) reports that the anti-inflammatory effect of fish oil appears to be due to powerful anti-inflammatory compound called resolvin (resolution- phase interaction product) E1, which is produced from EPA. Aspirin triggers the conversion of EPA to various resolvins and the workers identified resolvin E1 in the plasma of human subjects given ω-3 fatty acids and
aspirin. Resolvin E1 was discovered in vivo during the resolution phase of inflammation in exudates from inflamed tissues in a mouse model. The main task of resolvin E1 appeared to be to serve as a counter-regulator to proinflammatory mediators and turn down acute inflammatory processes before too much damage is done to normal tissue. The resolution of inflammation is said to be an active process controlled in part by endogenous chemical mediators that counter regulate proinflammatory gene expression and cell trafficking as well as stimulate inflammatory cell clearance. Their study showed that as little as 100 ng/mouse of synthetic resolvin E1 could decrease leukocyte infiltration into inflammatory loci by 50% to 60% in a mouse model of tumor-necrosis-factor (TNF)-α-induced inflammation. By comparison, local administration of dexamethasone produced 60% inhibition in this model, aspirin produced 70% inhibition, and indomethacin gave 25% inhibition.

The ADP induced platelet aggregation was significantly less in the cuttlefish liver oil fed animals compared to the normal group. This finding is in accordance with the earlier studies with marine fish oils (Hornstra et al., 1979; Needleman et al., 1980; Siess et al., 1990). Under normal circumstances, when linoleic acid (18:2 n-6) is the predominant PUFA in the diet, platelet aggregation and blood clotting are thought to be controlled by the opposing effects of thromboxane A2 (TXA2) produced by platelets and prostacyclin (PGI2) synthesized in vessel walls. TXA2 strongly promotes platelet aggregation and blood clotting, whereas PGI2 has the opposite effect. The EPA in fish oil can serve as a precursor of TXA3 and PGI3, where the TXA3 does not induce aggregation of platelets (Dyerberg and Jorgensen, 1980), whereas PGI3 like PGI2 is a potent antiaggregating agent (Hornstra et al., 1981). EPA has a high binding efficiency for platelet cyclooxygenase and thus competes with AA, inhibiting its conversion to TXA2 and PGI2 (Hamberg, 1980). 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 (Gryglewski et al., 1979; Whitaker et al., 1979). In conclusion, as reported in the case of marine fish oils the EPA content of the cuttlefish liver oil can be said to be the cause of the presently observed beneficial effects of feeding low dose of cuttlefish liver oil on suppression of inflammatory response and inhibition of platelet aggregation in rats.
CHAPTER 6
IMMUNOSTIMULATORY EFFECT
OF CUTTLEFISH LIVER OIL