Anti-obesity potentiality of Docosahexaenoic Acid (DHA) isolated from Topse fish oil (*Polynemus paradiseus*) on pre adipocyte cell line (3T3L1)

Project Submitted to Midnapore City College for the Partial Fulfilment of the Degree of
Master of Science (Food Science and Nutrition)

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Declaration

We do hereby declare that the present project work entitled 'Anti-obesity potentiality of Docosahexaenoic Acid (DHA) isolated from Topse fish oil (*Polynemus paradiseus*) on pre adipocyte cell line (3T3L1)' embodies the original project work carried out by us in the Department of Biological Sciences, Midnapore City College, Paschim Medinipur, West Bengal, India under the supervision of Dr. Shrabani Pradhan, Assistant Professor in Nutrition, Dept. of Paramedical and Allied Health Sciences, Midnapore City College, Kuturiya, P.O. Bhadutala, Pin-721129, Paschim Medinipur, West Bengal, India. No part thereof has been submitted for any degree or diploma in any University.

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Approval Sheet

This project report entitled **'Anti-obesity potentiality of Docosahexaenoic Acid (DHA) isolated from Topse fish oil (*Polynemus paradiseus*) on pre adipocyte cell line (3T3L1)'** by Laboni Majumder, Mamata Maity, Manik Hati & Rajat Kumar Barik is approved for the degree of M. Sc. in Food Science and Nutrition.

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(Name: Dr. Pradip Ghosh)

Date: _____

Place: _____

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Abstract

Docosahexaenoic acid (DHA, C22:6), a (n-3) fatty acid in fish oil, has been shown to decrease body fat and fat accumulation in rodents. We investigated the direct effect of DHA on cell growth, differentiation, apoptosis, and lipolysis using 3T3-L1 adipocytes. The rapidly rising prevalence of obesity, worldwide, has prompted re-evaluations of the definitions and diagnostic criteria, and of the extent of the burden it contributes to health care services. Although categorized arbitrarily for epidemiological purposes according to BMI > 25 kg/m2 ('overweight') and BMI > 30 kg/m2 ('obese'), the disease itself (ICD code E.66) is the process of excess fat accumulation. Obesity is the result of chronic energy imbalance in a person who consistently takes in more calories from food and drink than are needed to power their body's metabolic and physical functions. The obesity epidemic can be considered a collective response to this environment. Obesity is an important public health problem because it increases the risk of developing diabetes, heart disease, stroke, and other serious diseases. GC-MS study clearly revealed the presence of sixty-one fatty acids. Among the total fatty acids thirty- six are saturated fatty acids, fourteen are polyunsaturated fatty acids, and eleven are monounsaturated fatty acids. Also, physicochemical tests are done. Lipid accumulation in DHA treated group was confirmed by staining of Oil Red O, which exhibited a dramatic decrease in DHA treated group rather than control. We investigated the effect of DHA on adipogenesis related gene expression in 3T3-L1 adipocytes such as leptin, adiponectin, FAS (Fatty Acid Synthase), PPAR-α and inflammatory marker IL-6. Consistent with the decreases in TG accumulation, DHA significantly decreased the mRNA expression of leptin, FAS, IL-6 and increase the mRNA expression of adiponectin, PPAR-α in a dose dependent manner. By ELISA assay the effects of dietary fatty acids on cytokine production by adipocytes, the effects of anti-inflammatory cytokine, IL-1ra, IL-10 and inflammatory cytokine TNF-α were assessed.

Keywords: Docosahexaenoic acid, obesity, GC-MS study, adipocytes, adipokines.

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Chapter 1: Introduction

1. Background:

Obesity is a complex, chronic disease with several causes that lead to excessive body fat and sometimes, poor health. Body fat itself is not a disease, of course. But when your body has too much extra fat, it can change the way it functions. These changes are progressive, can worsen over time and they can lead to adverse health effects. Metabolic syndrome is characterized by a constellation of comorbidities that predispose individuals to an increased risk of developing cardiovascular pathologies as well as type 2 diabetes mellitus. The gut microbiota is a new key contributor involved in the onset of obesity-related disorder. Obesity has recently emerged as an imminent global public health concern over the past several decades. Recent reports from the Centres for Disease Control and Prevention estimate that two-thirds of the adult population and one-third of children and adolescents in the United States are overweight or obese. A combination of lack of physical activity and an increasingly unhealthy food environment is likely responsible. The etiology of obesity throughout time has focused on several key hypotheses with the thrifty gene hypothesis being perhaps the most widely accepted in the past. This theory states that obesity may be the result of an adaptive trait developed over the course of human evolution in response to periods of food scarcity and famine. It has now become evident that obesity is characterized by the persistent and low-grade inflammation in the adipose tissue, and serves as an independent risk factor for many metabolic disorders such as diabetes and cardiovascular disease. High mobility group box-1 (HMGB1) participates in the pathogenesis of inflammatory diseases. (**Monsey et al., 2014).**

Obesity is no longer an endemic metabolic disorder restricted to high-income countries. Instead, overweight and obesity have become pandemic. Globally, more people are obese (BMI ≥ 30) than underweight (BMI ≤ 18.5) and the mean global BMI keeps continuously increasing linearly over time **(Lancet 2016)** Obesity, especially abdominal obesity, is the main inducer of disturbed glucose homeostasis, insulin resistance and ultimately diabetes mellitus, type 2. Total body fat and abdominal and epididymal fat mass were considerably reduced in rodents fed sources of (n-3) PUFA such as fish oil or perilla oil compared with lard or corn oil–fed groups **(Lancet 2016, Murray et al., 2020)**. The mechanisms for the decreased fat deposition by (n-3) PUFA are not fully understood. This effect was thought to result from limited adipose tissue hypertrophy through enhancing fat mobilization and inhibiting hepatic lipogenesis **(Blahovaet al.,2021; Bailey et al., 2017)** rather than lower energy intake. Adipocytes play a central role in maintaining lipid homeostasis and energy balance by storing triacylglycerol (TG) 6 or releasing

FFA in response to changes in energy demands. Obesity results from a sustained imbalance between caloric intake and energy expenditure processes. In a state of caloric excess, white adipose tissue (WAT) plays a critical role by storing the surplus energy in the form of triglycerides, white adipocytes increase in number and size, and subcutaneous and visceral adipose depots expand. When adipose tissue enlargement is no longer capable of buffering the excess nutrients, numerous interconnected cell and tissue abnormalities arise, mainly in the visceral depots **(Olefsky et al., 2010; Item et al., 2012; Johnson et al., 2013; Smorlesi et al., 2012).** Increased catabolism in the mitochondria of hypertrophic adipocytes leads to oxidative stress and production of free radicals **(Patti et al., 2010; Codoner-Franch et al., 2011).**
Polynemus paradiseus is a small-sized fish from sweet water and known as Mango fish. This fish is characterized by the presence of soft bones and amazing taste.

Fish oil comes from many types of fish. It is rich in two important omega-3 fatty acids called eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The benefits of fish oil seem to come from its omega-3 fatty acid content. The body doesn't produce many of its own omega-3 fatty acids. Omega-3 fatty acids reduce pain and swelling, and also prevent the blood from clotting easily. Some fish oil products are approved by the FDA as prescription medications lower triglycerides levels. Fish oil is also available as a supplement. FAO/WHO, the American Dietetic Association and American Heart Association now recommended dietary intakes for total n-3 PUFA of 1.4- 2.5 g/day, with EPA and DHA ranging from 140-600 mg/day, depending on the authority issuing the guidelines **(Ros et al., 2018)**. After few investigations between changes in body composition and supplementation of fish oil it controls the overweight and obese in adults and may help to reduce the waist hip ratio. Fish oil may reduce the belly fat by decreasing the fat cell size **(Garaulet et al, 2006)**.

1.1 National Scenario of Obesity:

India is the second most populous developing country with industrialization, and rapid urbanization has resulted in a significant number of people being overweight or obese and/or having elevated blood pressure and blood glucose. The prevalence and prevalence rate per 100,000 population were calculated at the national level and by age group, sex, and type of residence for each state and union territory. The national prevalence of overweight, obesity, hypertension, and diabetes were 14.6%, 3.4%, 5.2%, and 7.1%, respectively. The highest

prevalence of these conditions/diseases at the national level was seen among those aged 35–49 years (54 years for men), especially women living in urban areas **(Vennu et al.,2019)**

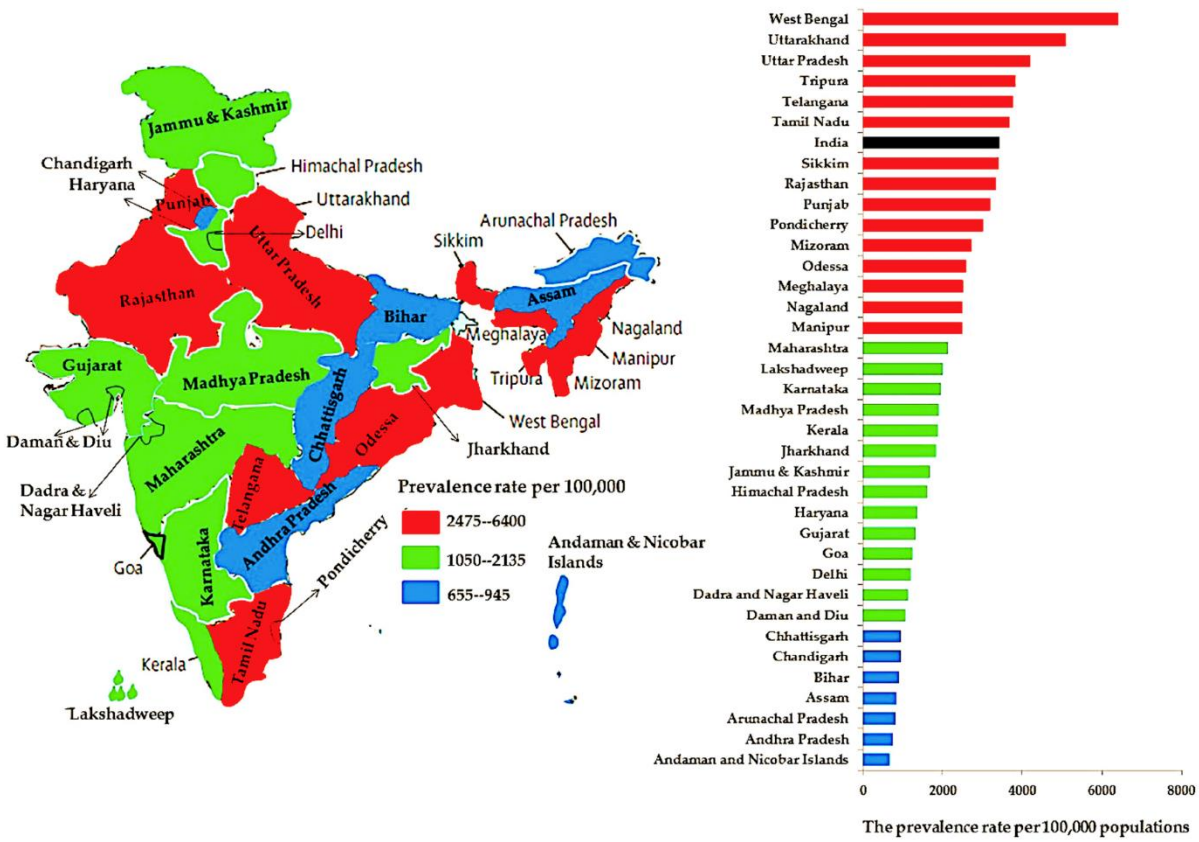

Figure: The prevalence rates of obesity among adults at the national level and per state and union territory (Vennu et al.,2019)

1.2. Pathophysiology of obesity:

Obesity is an exaggeration of normal adiposity and is a central player in the pathophysiology of diabetes mellitus, insulin resistance, dyslipidaemia, hypertension, and atherosclerosis, largely due to its secretion of excessive adipokines. Obesity is a major contributor to the metabolic dysfunction involving lipid and glucose, but on a broader scale, it influences organ dysfunction involving cardiac, liver, intestinal, pulmonary, endocrine, and reproductive functions. Inflammatory, insulin-resistant, hypertensive, and thrombotic-promoting adipokines, which are atherogenic, are counterbalanced by anti-inflammatory and anti-atherogenic adipocyte hormones such as adiponectin, vastatin, and acylation-stimulating

protein, whereas certain actions of leptin and resist in are pro-atherogenic. Adiponectin is protective against liver fibrosis due to its anti-inflammatory effect, whereas inflammatory cytokines such as tumour necrosis factor-α are detrimental for both fatty liver and pancreatic insulin release. Obesity contributes to immune dysfunction from the effects of its inflammatory adipokine secretion and is a major risk factor for many cancers, including hepatocellular, oesophageal, and colon. Because of the accelerating effects that obesity has on the worsening of metabolic syndrome and cancer, it has the potential to be profoundly detrimental to our species if major methods of prevention and/or effective treatment are not realized. It is essential then to institute major educational efforts aimed at promoting better eating habits and physical exercise. **(Schoonjans et al., 1996)**

1.3. Obesity and gene interaction:

Leptin is a peptide hormone which is coded for by the obese gene. Influences the quantity of food consumed relative to the amount of energy expended. When leptin levels are high, appetite is reduced and energy expenditures is increased. Main role of leptin is to achieve an energy balance in the body. Leptin binds to receptors in brain and performs several actions that may prove that leptin is important in treating obesity. This hormone is produced by the adipose tissue, mainly by the white adipose tissue of the human body. Researchers thought that leptin would be the key in controlling obesity. But research revealed that if greater amount of leptin release, then it is less effective in the brain for controlling hunger and food intake. The result is uncontrolled feeding, leading to greater food intake and fat storage. Typically, females have more leptin than males. **(Mahmoud et al., 2022)**

Adiponectin, also known as adipocyte complement-related protein of 30 kDa (Acrp30), was identified by different groups. Adiponectin is an adipokine abundantly produced and secreted by adipose tissues and widely recognized for its antidiabetic, anti-inflammatory, antiatherogenic, and cardioprotective effects. Adiponectin is a protein hormone of 244 amino acids that circulates in high concentrations (5–30 μg/mL) accounting for 0.01% of total serum proteins. Adiponectin expression and serum levels are decreased in obese patients, pigs, and rodents. Sexual dimorphism has been observed in adiponectin expression, with males showing lower levels than females. Recent data have established that the gene encoding TNF-a is expressed in white adipose tissue and that its level of expression (at least its mRNA) is increased in human obesity and rodent models of obesity. These results led to a hypothesis: that increased fat-derived TNF-alpha was a causative factor in the induction of insulin resistance and type 2 diabetes associated with obesity. **(Caselli, 2014)**

IL-10 is a regulatory cytokine with pleiotropic effects on numerous cell types that express IL-10 receptor 1 (IL10R1) and IL-10 receptor 2 (IL-10R2) and has an important and well-characterized role in regulating the immune response. Interleukin-10 (IL-10) is also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. IL-10 is an immunosuppressive cytokine produced by a variety of mammalian cell types including macrophages, monocytes, T cells, B cells and keratinocytes. Mature human IL-10 shares 72% - 86% amino acid sequence identity with bovine, canine, equine, feline, mouse, ovine, porcine, and rat IL-10. Whereas human IL-10 is active on mouse cells, mouse IL-10 does not act on human cells. IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNFα and GM-CSF made by cells such as macrophages and regulatory T-cells. It also displays a potent ability to suppress the antigen-presentation capacity of antigen presenting cells. However, it is also stimulatory towards certain T cells and mast cells and stimulates B cell maturation and antibody production. **(Esposito et al., 2003).**

Interleukin (IL)-6 levels are higher in obese compared with nonobese individuals. IL-6 levels are positively correlated with BMI and percent fat mass (PFM). Chronic exposure to elevated IL-6 levels is associated with the development of insulin resistance, the metabolic syndrome, and T2D. The proposed mechanism of this biological effect is reduction of glucose transporter-4 and insulin receptor substrate-1 expression in response to IL-6 exposure. IL-6 were found to be increased and correlated with measures of insulin resistance in male subjects with abdominal obesity. Weight loss was associated with reduction of plasma levels of TNF-α and IL-6 by 25–30%.IL-6 expression is increased in obese adipose tissue from obese individuals as compared to adipose tissue from lean individuals if normalized for the number of adipocytes. IL-6 receptor expression is increased in the hypothalamus, suggesting a possible role of IL6 in controlling appetite and energy intake. Circulating levels of IL-6 were found to be increased with increasing adiposity and to be associated with the development of obesity-related complications **(Timper et al., 2017).**

POMC is an appetite inhibitory gene found on chromosome 2 in humans. It influences the leptin–melanocortin system as a deficiency of the POMC protein causes an absence of ACTH and alpha-MSH, which are cleaved from the POMC protein. Hence, a deficiency of POMC leads to hyperphagia, lower resting metabolic rate, and resultant severe obesity with red hair and pale skin. **(Mahmoud et al., 2022).**

The melanocortin-4 receptor (MC4R) gene is now considered the most common associated gene for childhood obesity and found in about 4% of affected cases prior to advanced genetic

testing and next generation sequencing (NGS). It was first discovered to be related to body weight in 1998 and now multiple studies have investigated its mechanism and the function of different mutations. The MC4R gene codes for the MC4R protein, which plays an important role in energy homeostasis and food intake behaviour. The central melanocortin pathway regulates energy balance and homeostasis by activating or inhibiting leptin and its receptor is mediated by two subsets of neurons as well as MC3R and MC4R in the arcuate nucleus of the hypothalamus. **(Ayers et al., 2018).**

FTO was the first obesity-susceptibility gene discovered through GWAS in European patients with type 2 diabetes **(Frayling, Timpsonet al., 2007).** Multiple single nucleotide polymorphisms (SNPs) in the first intron of the gene have shown a significant association with type 2 diabetes. However, after controlling for BMI, there was no association with type 2 diabetes, thereby suggesting that the FTO and type 2 diabetes association was mediated through FTO's effect on BMI. Another study was conducted in Setardinian patients and confirmed the same results. The rs9939609 and rs9930506 SNPs were identified in FTO with significant association with BMI **(Scuteri, Sanna et al., 2007)**; **(Dina, Meyre et al., 2007).** Other GWAS studies in European populations have reported several other SNPs located in the same chromosomal location. In addition, significant association between FTO SNPs (rs9939609, rs17817449, rs12149832) and BMI was reported in three large studies conducted in Asian populations **(Mahmoud et al., 2022).**

Peroxisome Proliferator Activated Receptors (PPARs), a group of ligand-activated transcription factors that consists of three members: PPAR-α, PPAR-β and PPARɣ. Each PPAR governs the expression of specific sets of target genes involved in various cellular processes ranging from inflammation to glucose and lipid metabolism. Currently, synthetic agonists for PPARs are prescribed for the treatment of dyslipidaemia and insulin resistance. Apart from metabolic abnormalities, obesity is also accompanied by a chronic low-grade inflammation, which is generally believed to originate from expanding adipose tissue. Elevated secretion of pro-inflammatory factors from adipose tissue has been linked to the development of atherosclerosis and insulin resistance. Since PPARs have important anti-inflammatory properties in a wide variety of cell types, they might protect against obesity induced inflammation and its complications **(Stienstra, 2007).**

1.4 Role of DHA in obesity prevention:

Healthy diet and correct lifestyle play crucial role for the treatment of obesity. Obesity is significantly prevalent in western countries. Particularly low basal docosahexaenoic acid

(DHA) levels, appear to be important in increasing cancer risk and its relapse, influencing its progression and prognosis and affecting the response to treatments. On the other hand, DHA supplementation increases the response to anticancer therapies and reduces the undesired side effects of anticancer therapies. Experimental and clinical evidence shows that higher fish consumption or intake of DHA reduces the risk of obesity. Controversy exists on the potential anticancer effects of marine ω-3 PUFAs and especially DHA, and larger clinical trials appear mandatory to clarify these aspects. DHA is essential for the growth and functional development of the brain in infants, and is also required for maintenance of normal brain function in adults. **(Rezzo et al., 2012).** Nevertheless, a randomized trial in healthy young adults, providing increasing doses of EPA + DHA up to 1.8 g/ day for five months, found a marginal decrease in TNF- α levels and no change in IL-6 levels, documenting no effect of marine PUFAs on circulating inflammatory molecules. However, we acknowledge that healthy adults are unlikely to have high levels of inflammatory markers, hence less likelihood of showing a response. In this light, we found several positive results, when considering the effects of omega-3 fatty acids administration on inflammation associated with several diseases, including obesity. **(Alessio et al., 2016)** Docosahexaenoic acid (DHA, C22:6), a (n-3) PUFA in fish oil, inhibits the proliferation not only of various cancer cells but also of normal cells. Several mechanisms have been suggested for the antiproliferative effects on tumour cells, including peroxidative damage from lipid peroxidation **(Dommels et al., 2003; Chajes et al., 1995)** apoptosis induction **(Merendino et al., 2003)**,modulation of eicosanoids **(Connolly et al., 1999)** and increasing membrane permeability to increase susceptibility to anticancer drugs **(Stillwell et al., 1993)** Interestingly, apoptosis in adipose tissue has been described **(Prins et al., 1994)** and it has been suggested that adipocyte deletion by apoptosis could be a contributor to body fat loss **(Kim et al., 2006)** The purpose of this study was to examine the potential of DHA to function as an anti-obesity agent via modulation of adipocyte lipid storage and/or preadipocyte cell proliferation. It specifically explored the effects of DHA on 3T3-L1 preadipocyte differentiation, lipolysis, and apoptosis. The determination of EPA and DHA in fish oil has been reported by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). However, the HPLC is not sensitive enough and the cost of LC-MS is high. In the present study, a new gas chromatography–mass spectrometry (GC-MS) method was developed for the analysis of fish oil **(Yi et al., 2014).**

Chapter 2: Literature Review

2. Review & Literature:

Siroma et al., 2022 established the role of ingestion of polyphenols and omega-3 polyunsaturated fatty acids (ω-3 PUFAs) in reducing obesity and related metabolic diseases (RMDs). The consumption of vegetables, fish and by-products rich in polyphenols and α-linolenic acid (ALA), as well as oils rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are associated with a decrease in obesity and its RMDs in consumers.

Verduci et al., 2021 revealed preclinical and clinical works on factors that may promote BAT or browning of white adipose tissue (WAT) from fatal age to adolescence. Maternal lifestyle, type of breastfeeding and healthy microbiota can affect the thermogenic activity of BAT. Environmental factors such as exposure to cold or physical activity also play a role in promoting and activating BAT.

Balakrishnan et al., 2021 evaluated the bioavailability of DHA, mechanisms of DHA transport, and role of DHA in preventing neurodegenerative disorders, which provides better insight into the pathophysiology of these disorders and use of structured DHA in improving neurological health.

Pradhan et al., 2020 extracted and characterized the Tapra fish oil as well as applied it to evaluate anti-obesity potentiality. Gas Chromatography-Mass Spectrometric analysis clearly revealed the presence of nine different fatty acids. When the fish oil was applied to high-fat diet-induced obese mice, it showed significant reduction of body weight, Body Mass Index, and serum lipid profiles compared to the high-fat diet induced obese mice. The levels of leptin and TNF-α were moderately reduced in fish oil treated high-fat diet-induced obese mice than control obese mice. In conclusion, the Tapra fish oil was enriched with essential fatty acids and it could be used as an anti-obese food supplement.

Silva et al., 2017 revealed that the consumption of unsaturated fatty acids, MUFA, and PUFA, and especially EPA and DHA, which can be applied as food supplements, may promote effects on glucose and lipid metabolism, as well as on metabolic inflammation, gut microbiota, and hepatic metabolism**.**

Simopoulos, 2016 suggested that omega-6 and omega-3 fatty acids elicit divergent effects on body fat gain through mechanisms of adipogenesis, browning of adipose tissue, lipid homeostasis, brain-gut-adipose tissue axis, and most importantly systemic inflammation. A

balanced omega-6/omega-3 ratio is important for health and in the prevention and management of obesity.

Molfino et al., 2016 explored the capacity of DHA in controlling obesity-related inflammation and in reducing insulin resistance in BC development, progression, and response to therapies. DHA supplementation increases the response to anticancer therapies and reduces the undesired side effects of anticancer therapies.

Huang et al., 2016 explored the physiology of n-3 PUFA effects in the body is delineated from studies conducted in both human and animal experiments. Although mechanistic studies in human are limited, numerous studies conducted in animals and models in vitro provide potential molecular mechanisms of the effects of these fatty acids.

Fernández et al., 2015 focused on the role of n-3 PUFAs-derived specialized pro resolving lipid mediators such as resolvins, protectins and maresins. The effects of n-3 PUFAs on adipose tissue glucose uptake and insulin signalling are also summarized. They focus in reviewing the current knowledge about effects of marine n-3 PUFAs on adipose tissue metabolism and secretory functions.

Lorente-Cebrián et al., 2013 illustrated current knowledge about the efficacy of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) in treating/preventing several metabolic pathologies. They reviewed systematically the published evidence on the effectiveness of n-3 LC-PUFAs fish consumption or n-3 LC-PUFAs supplementation on prevention/treatment of obesity, metabolic syndrome, and cardiovascular diseases.

Flock et al., 2013 assessed the role of EPA and DHA in ameliorating obesity-induced inflammation, evaluating clinical evidence and mechanisms of action. EPA and DHA may improve insulin sensitivity by generating pro resolving lipid mediators and promoting alternatively activated macrophages.

Banz et al., 2012 suggested a protective role of SDA consumption on markers of dyslipidaemia and inflammation. The AHA recommends that healthy individuals consume oily fish at least twice per week and individuals with a history of cardiovascular disease consume 1 g of EPA+DHA/day.

Chapter 3: Aims and Objectives

3. Aims & Objectives:

Objectives are,

- To isolate and characterize the potent fatty acid from marine *Polynemus paradiseus* oil.
- To analyse the anti-obesity potentiality of potent fatty acid on 3T3L1 cell line.
- To establish the anti-obese drug by using isolated fatty acid assessing genomic marker.

Ultimately the aim is,

to study the effectiveness screened fatty acid from marine *Polynemus paradiseus* oil on obesity.

Chapter 4: Materials and Methods

4. Materials and Methods:

4.1. Sample collection: At first the different varieties of *Polynemus paradiseus* will be collected from coastal area of Purba Medinipur district, West Bengal.

4.2. Extraction of Fish Oil: At first 10g of different sea fish, *Polynemus paradiseus* dust mixed with hexane and isopropanol at 3:2 ratio. Then it was kept in shaker for overnight. After that the mixing solution kept in Soxhlet for 6 hours and then centrifuged at 5000rpm for 20minutes. After that collect the supernatant and then filter it using filter paper. Then the filtrate solution was poured into rotary evaporator for evaporation of the volatile compounds and the fish oil was collected for further analysis **(AOCS 1994)**.

4.3. Fatty acid analysis from *Polynemus paradiseus* oil by GC-MS: Extraction of the fish lipids will be done according to the method of AOAC-23 with some modification by Frankel et al., 1993 by using institute Research laboratory facility.

4.4. Cell Culture: 3T3L1 is a pre-adipocytes cell. After confluency of this cell by adding growth media with 3nM insulin (Humulin R; Eli Lilly), 0.25 µM dexamethasone (Sigma-Aldrich) and 0.5mM 1-methyl-3-isobutyl-xanthine (Sigma-Aldrich). From day 3 until day 7, cells will be maintained in growth media supplemented with 3 nM insulin after which the mature adipocytes will be maintained in growth media. For obesity study treatment by *Polynemus paradiseus* oil, pre-adipocytes will be incubated from day 8 to 9. After further experiment cell will be analysis for different gene expression.

4.5. RNA isolation and PCR: To check the gene marker by semi-q- PCR and Agarose Gel Electrophoresis using TNF-α, IL-6, PPAR-α, PPAR-Ɣ, PPAR-β, IL-1ra, leptin, adiponectin, FAS, LPL, CPT 1, SREBPs. RNA isolation from obese cell and different gene expression followed by PCR and RT-PCR. Total RNA will be extracted from the obese cell using carried out following the protocol of HiPurA Total RNA Miniprep purification kit (Hi-Media Hi-GenoMB, Maharashtra, India) after that reverse transcription reaction mixture will be prepared according to Hi-cDNA synthesis kit (Himedia, HiGenoMB, Maharashtra, India). A published house-keeping primer set will be used for GAPDH(sense: GGTGAAGGTCGGAGTCAACG, antisense: GTGAAGACGCCAGTGGACTC) TNF-αprimerset (sense: TTCTGTCTACTGAACTTCGGGGTGATCGGTCC, antisense: GTATGAGATAGCAAATCGGCTGACGGTGTGG)IL-1Ra primer set (sense:

GCAGCACAGGCTGGTGAATGAC antisense: TGCCCCCGTGGATGCCCAAG) IL1b primer set (sense: ATGGCAACTGTTCCTGAACTCAACT antisense: CAGGACAGGTATAGATTCTTTCCTTT) IL1R1 primer set (sense: GGTGCCTCTGCTGTCGCTGG antisense: CGCTGTGGGAAGGTGGCCTG) forward and reverse primer sequences designed for leptin (NM_000230.2) are (5′ to 3′) TCCCCTCTTGACCCATCTC and GGGAACCTTGTTCTGGTCAT, respectively. Next PCR will perform as follow: initial denaturation of DNA at 95˚c for 1min, denaturation at 95˚C for 30 sec, DNA annealing at 65˚c for 45sec and extension of DNA at 72˚C for 2 min, final extension of DNA at 72˚c for 10min. the second and third step were repeated for total of 42 thermal cycles. Lastly the PCR tube with sample was hold at 4˚c.

4.6. Adipocyte cell staining by Oil Red O: Oil Red O is a fat-soluble diazo dye used for lipid and fat deposits in cell and tissue. After applying the Oil Red O stain, it will give red colour to the fat cells. That can be differentiate the adipocyte cell from normal cell **(Pradhan et al., 2020)**.

4.7. Gene expression study: To check the gene markers such as leptin, adiponectin, FAS, LPL, CPT 1, TNF-α, IL-6, PPAR-α, PPAR-ɣ, PPAR-β, IL-1ra, IL-10 by semi-q- PCR **(Arai et al., 2009).**

4.8. ELISA assay: We will do **ELISA** to check the expression of leptin, adiponectin, FAS, LPL, CPT 1, SREBPs, TNF-α, IL-6, PPAR-α, PPAR-ɣ, PPAR-β, IL-1ra.

4.9. Statistical Analysis: Analysis of somatic body mass index and then statistical analysis were done by ANOVA followed by multiple two-tail t-test and data with different superscripts (a, b, c) in a specific vertical column differ from each other significantly ($p< 0.05$).

Chapter 5: Results

5. Results:

5.1. GC-MS chromatograms of oil extracted from *Polynemus paradiseus* oil:

GC-MS study clearly revealed the presence of sixty-one fatty acids. Among the total fatty acids thirty- six are saturated fatty acids, fourteen are polyunsaturated fatty acids, and eleven are monounsaturated fatty acids. (Fig 1 and Supplementary Table 1).

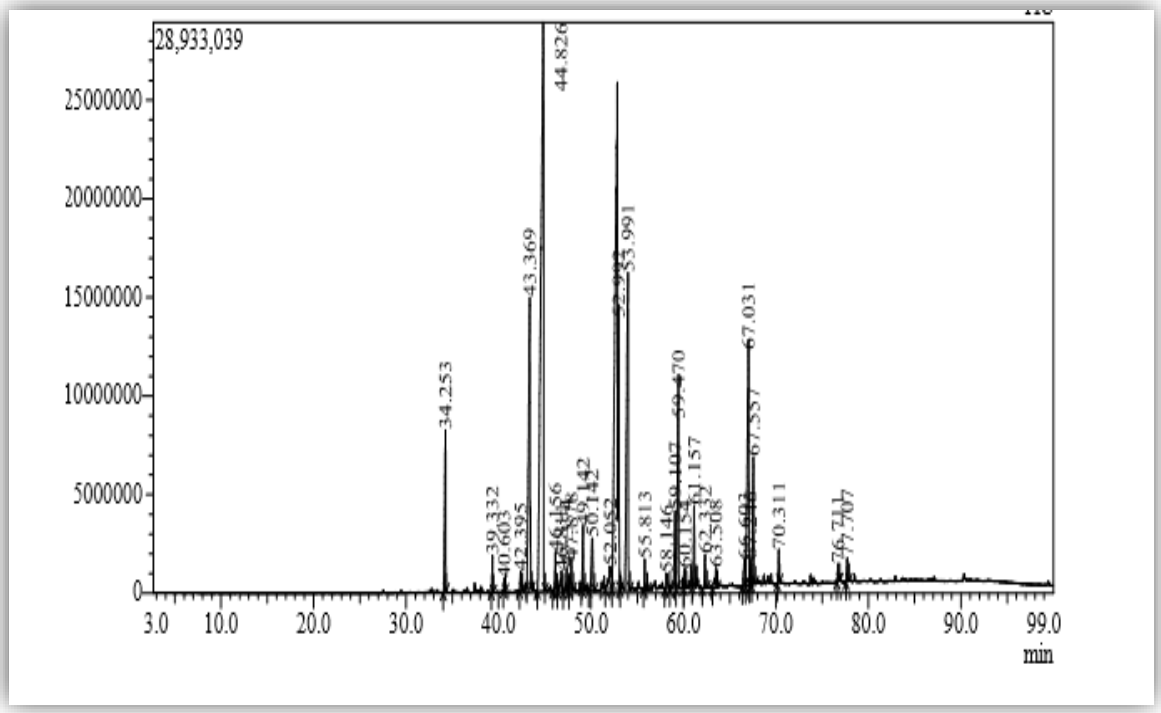

Figure 1: Gas chromatography-mass spectrometer (GC-MS) chromatograms of *Polynemus paradiseus* oil.

Table 1: Fatty acids profiling of oil by *Polynemus paradiseus* using Gas chromatography-mass spectrometer (GC-MS).

Polyunsaturated fatty acids (PUFAs)	Monounsaturated fatty acids (MUFAs)
Methyl gamma-linolenate	Methyl palmitoleate (Palmetoleic acid); Hexadec-9-enoic acid
Octadeca-6,9,12-trienoic acid	Methyl cis-vaccenate
Methyl linolenate	(Z)-octadec-11-enoic acid
(9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid (Linolenic acid)	Methyl cis-10-heptadecenoate
Methyl linoleate (linoleic acids)	Heptadec-10-enoic acid
Methyl linolelaidate	Methyl cis-11-icosenoate
(9E,12E)-octadeca-9,12-dienoic acid (Linoeladic acid)	Icos-11-enoic acid or gondoic acid
Methyl arachidonate	Methyl cis-12-Octadecenoate
(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid	(Z)-octadec-12-enoic acid (Petroselinic acid)
Methyl cis-7,10,13,16,19-docosapentaenoate	Methyl cis-11-icosenoate
(7Z,10E,13E,16E,19E)-docosa-7,10,13,16,19-pentaenoic acid	Methyl cis-12-Octadecenoate
Methyl cis-5,8,11,14,17-Eicosapentaenoate or Icosa-5,8,11,14,17-pentaenoic acid	
Methyl cis-4,7,10,13,16,19-Docosahexaenoate	
Docosa-4,7,10,13,16,19-hexaenoic acid	

5.2. Physicochemical characteristics of *Polynemus paradiseus* oil:

The acid and peroxide indexes evaluate the oil's quality. The acid value was 4.53 ± 0.23 mg KOH/g, which is within the range of 5.0 mg KOH established by the National Agency of Sanitary Surveillance (Muniz et al., 2015) and the peroxide value was 3.66 ± 0.33 mEq/kg that is within the range of 5.0 milliequivalent of active oxygen/kg oil established by Codex Alimentarius Commission, WHO (2017). The saponification value was 135.52 ± 2.68 mg KOH/g which implies that the oil comprised a large proportion of polyunsaturated fatty acids (Ferreira et al., 2006). The totox value was 8.71 ± 0.15 mg which was within the range of the National Agency of Sanitary Surveillance (Ferreira et al., 2006) (Table 2).

Table 2: Physicochemical Properties of *Polynemus paradiseus* oil

Value	**Mean ±SE**
Acid value (mg KOH/g)	4.53±0.23
Saponification value (mg KOH/g)	135.52±2.68
Peroxide value (mEq/kg)	3.66±0.33
Totox value	8.71±0.15

5.3. Effect of docosahexaenoic acid (DHA) supplementation on lipid accumulation and morphological changes in 3T3-L1 adipocytes:

According to our research, the DHA-treated group had pronounced morphological alterations and a considerable reduction in the size and quantity of lipid droplets. Lipid accumulation in DHA treated group was confirmed by staining of Oil Red O, which exhibited a dramatic decrease in DHA treated group rather than control. As per Figure 2, out of the four doses, 3.75 µM/ml DHA was the most effective in preventing adipocyte development, indicating that it has antiadipogenic effects.

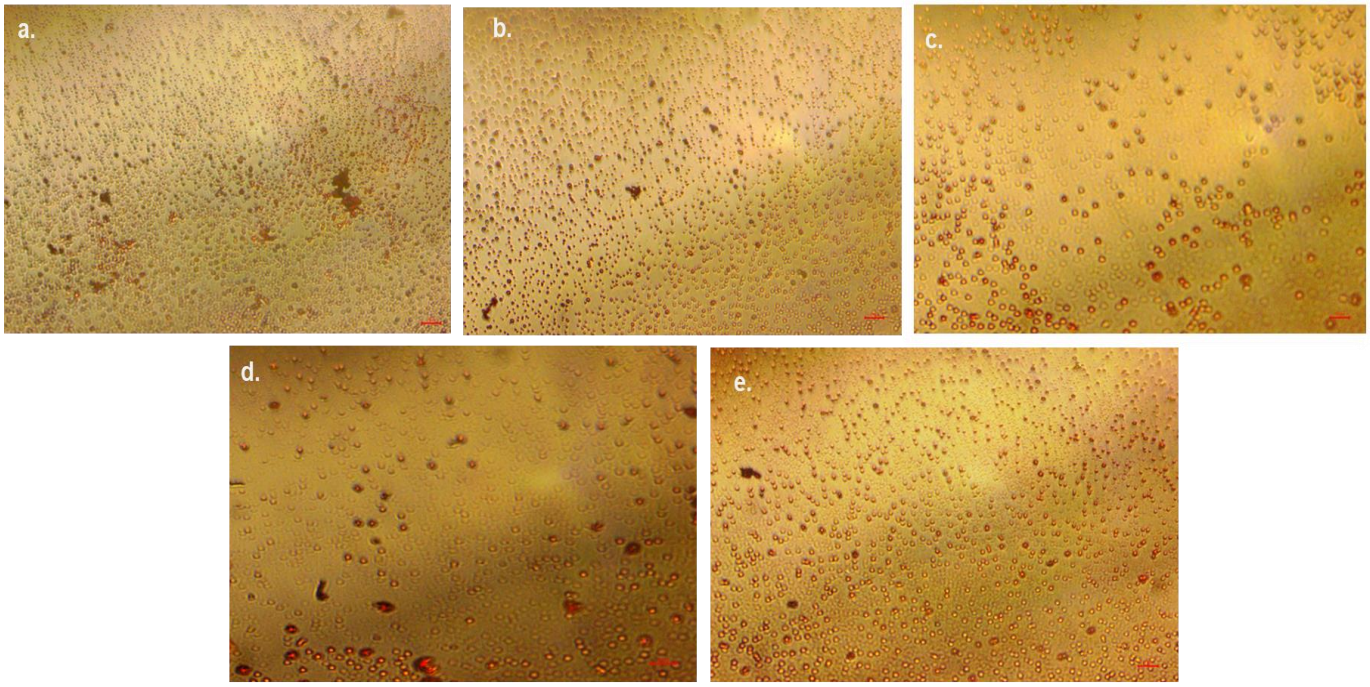

Figure 2: Oil Red O staining at different doses of docosahexaenoic acid (DHA) on 3T3-L1 cell line. (a) Control (b)1.25 µM, (c)2.5 µM, (d) 3.75 µm & (e) 5 µM.

5.4. Effect of DHA on adipogenesis related gene expression in 3T3-L1 adipocytes:

To gain a better understanding of the molecular mechanism(s)under lying the anti-adipogenicity effects of DHA, we have tested different obesity related genes such as leptin, adiponectin, FAS (Fatty Acid Synthase), PPAR-α and inflammatory marker IL-6. Consistent with the decreases in TG accumulation, DHA significantly decreased the mRNA expression of leptin, FAS, IL-6 and increase the mRNA expression of adiponectin, PPAR-α in a dose dependent manner. In this study, cells were exposed to DHA at various concentrations (1.25, 2.5, 3.75, and 5 µM). Leptin was increased in 2.5 and 3.75 µM dose treatment than control by 1.06-fold. Expression level of adiponectin was increased by 2.16-fold and 1.65-fold, after treatment with 2.5 µM and 3.75 µM doses of DHA. The inflamatory marker, IL-6 was found decreased by 1.19 fold after tratement of 3.75 µM dose of DHA. We also measured the expression of peroxisome proliferator-activated receptor-α (PPAR-α) and FAS gene.Expression of PPAR-α level was increased 1.24-fold and FAS level was dereased by 1.12 fold after treatment of DHA.

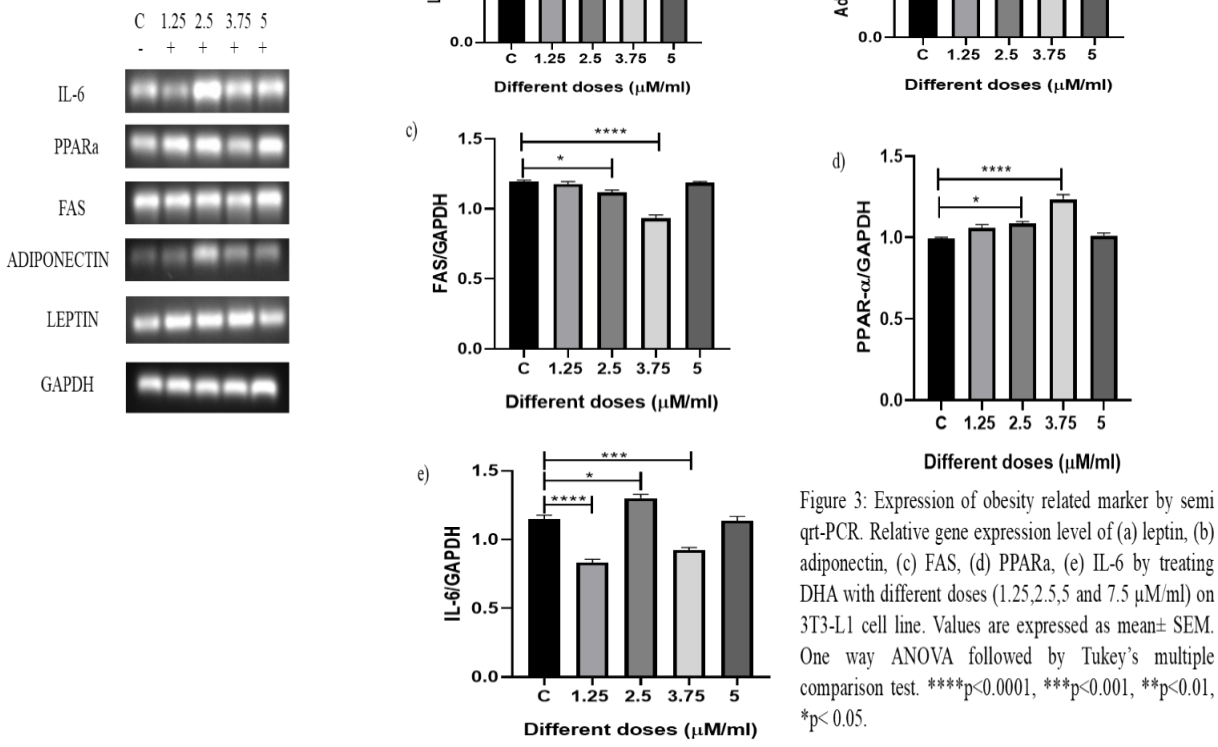

Figure 3: Expression of obesity related marker by semi qrt-PCR. Relative gene expression level of (a) leptin, (b) adiponectin, (c) FAS, (d) PPARa, (e) IL-6 by treating DHA with different doses (1.25,2.5,5 and 7.5 µM/ml) on 3T3-L1 cell line. Values are expressed as mean± SEM. One way ANOVA followed by Tukey's multiple comparison test. ****p<0.0001, ***p<0.001, **p<0.01, *p< 0.05.

5.5. ELISA assay:

To further investigate the effects of dietary fatty acids on cytokine production by adipocytes, the effects of anti-inflammatory cytokine, IL-1ra, IL-10 and inflammatory cytokine TNF-α were assessed. Here, cells were treated with different doses (1.25, 2.5, 3.75 & 5 µM) of DHA. Among the all doses, treatment with 3.75 µM/ml DHA shown more effective result. Increased IL-1ra expression by 30.07%, 42.99% after treating 2.5 & 3.75 µM doses of DHA. IL-10 expression increased by 39.64% and 46.16% following treatment with 2.5 & 3.75 M dosages, which may have activated anti-inflammatory signalling pathways through its contact with the IL-10 receptor and subsequently had an anti-inflammatory effect (Lumeng et al., 2007). TNF-α markers was downregulated by 7.38% and 13.06% respectively.

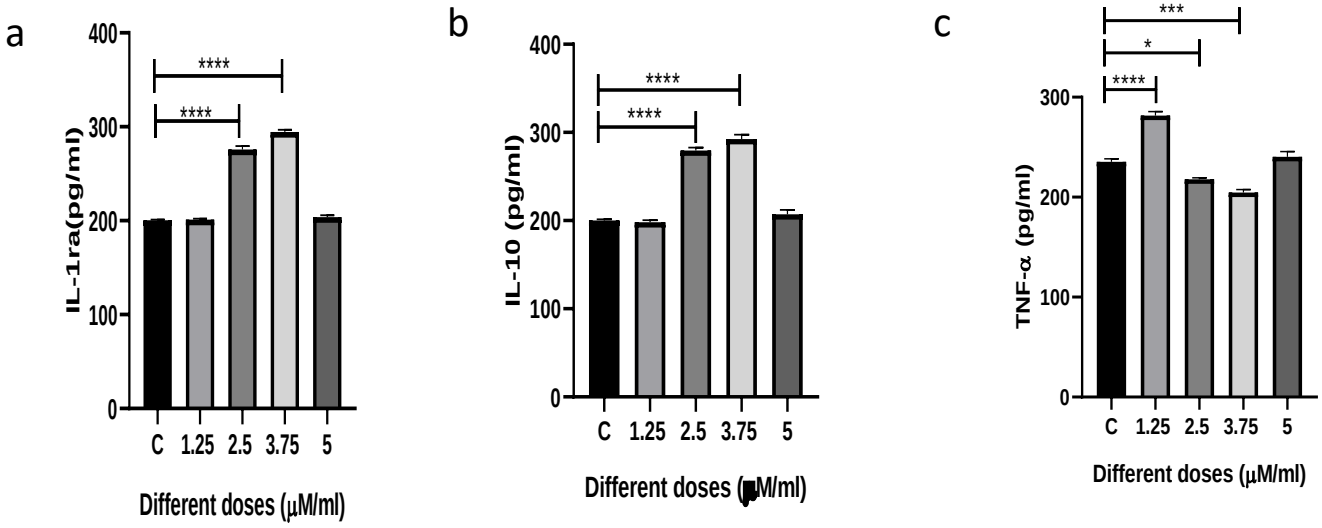

Figure 4: Effect of docosahexaenoic acid (DHA) on secretion of IL-1ra, IL-10, TNF-α by 3T3-L1 adipocytes. Differentiated adipocytes treated with different doses of docosahexaenoic acid (DHA) (1.25,2.5,5 and 7.5 µm/ml) for 24 hours. Values are expressed as mean± SEM. One way ANOVA followed by Tukey's multiple comparison test. ****p<0.0001, ***p<0.001, **p<0.01, *p< 0.05.

Chapter 6: Discussion

6. Discussion:

Polynemus paradiseus is a common fish which largely available in sea coastal area of West Bengal region. It is a locally available indigenous food which easily affordable for all community. *Polynemus paradiseus* contain several conjugated fatty acids which have beneficial effect in our health. Considering the different health beneficial effects of fish oil as suggested earlier by **Calder (2017)**, in this study, we extracted and characterized the *Polynemus paradiseus* oil and evaluated its effect on obesity. The low acid values determined for *Polynemus paradiseus* oil indicated that the triacylglycerol had not been hydrolysed, which could indicate good stability. The higher saponification value of fish oil indicated the high content of medium chain fatty acids. The lower peroxide value indicated a good quality of oil and a good preservation status. The range of totox value showed the presence of high primary and secondary oxidative stability **(Pradhan et al., 2020)**.

It has been demonstrated that the (n-3) fatty acid docosahexaenoic acid (DHA, C 22:6) found in fish oil reduces body fat and fat formation in mice. Recent years have seen a rise in interest in the effects of DHA on the metabolism of adipose tissue and their prevention of obesity **(Buckley & Howe 2010)**. Differentiated 3T3-L1 adipocytes are a widely used *in vitro* model of white adipocytes. Using 3T3-L1 adipocytes, we examined the direct impact of DHA on cell proliferation, differentiation, apoptosis and lipolysis **(Morrison & McGee 2015)**. In this study we evaluated the presence of neutral lipids by Oil Red O staining. Oil Red O staining was used as an indicator of adipogenesis. Here, cells were treated with different doses (1.25 µM/ml, 2.5 µM/ml, 3.75 µM/ml & 5 µM/ml) of DHA. DHA treatment during differentiation suppressed lipid accumulation, as shown by dose- dependent decreases droplet size and percentage of lipid area. Among the all doses we found 3.75 µM/ml dose was more effective than other doses. These results demonstrate that DHA may exert its anti-obesity effect by inhibiting differentiation to adipocytes, inducing apoptosis in post confluent preadipocytes and promoting lipolysis **(Kim et al., 2007)**.

Leptin play important role to regulates hepatic lipogenesis by suppressing the expression of key enzymes in the fatty acid synthesis pathway **(Cohn et al., 2002)**. White adipose tissue produces leptin, which binds to receptors in the brain and delivers signals to control food intake and energy expenditure **(Izquierdo et al., 2019)**. In this study, mRNA and protein expression of leptin were decreased after DHA treatment by 1.06-fold, which may be correlated with the inhibition of fat accumulation and adipocyte proliferation at 2.5 and 3.75 µM doses by disrupting the leptin signaling as suggested by **Izquierdo et al. (2019)**. Thereby, adipose FASN

mRNA expression is significantly related to obesity, predominantly visceral fat accumulation, impaired insulin sensitivity and circulating adipokines. Moreover, FAS was overexpressed by 1.09-fold at 2.5 µM dose in adipose tissue and decreased by 1.12-fold at 3.75 µM dose. DHA treatment with different doses (1.25, 2.5, 3.75 & 5 µM) progressed the mRNA and protein expression level of PPAR-α by 1.24-fold and 1.18-fold, respectively. Additionally, PPAR- may be essential for the removal of apolipoproteins for triglycerides and cholesterol, which may aid to prevent various cardiovascular diseases **(Blaschke et al., 2006)**. Adiponectin is a fat-derived hormone that play an important role in maintaining against insulin resistance or diabetes and atherosclerosis. Decreased levels of adiponectin is the central role in the development of type 2 diabetes, obesity and cardiovascular disease in humans **(Achari et al., 2017)**. Moreover, the adiponectin expression was advanced by 2.16-fold and 1.65-fold respectively with the treatment of 3.75 µM and 2.5 µM dose DHA. It might be due to the presence of unsaturated fatty acids which influence adiponectin circulation to control obesity **(Gammelmark et al., 2012)**. In obese patients IL6 serum level was found to be increased that resulting the development of chronic inflammatory conditions and serum lipid concentrations abnormalities **(Galcheva et al.,2011; Stenlöf et al.,2003)**. Moreover, increased IL-6 levels in individuals with obesity may result in increasing the risk of cardiovascular complication, insulin resistance, and type 2 diabetes **(Takumansang et al., 2013)**. The inflamatory marker, IL-6 was found decreased by 1.19 fold after tratement of 3.75 µM dose of DHA.

In this study, we used four different doses of DHA on 3T3-L1 cell line, among the doses 3.75 µM dose exerts most effective result on IL1-ra, IL-10 and TNF-α. The interleukin-1 (IL-1) family of cytokines and receptors are key mediators of innate inflammatory responses and exhibit both pro- and anti-inflammatory functions. IL-1ra is positively correlated with body mass index (BMI) and insulin resistance and plays a role in cholesterol excretion. Supplementation of DHA can increase IL-1ra than control. IL-10 is an anti-inflammatory cytokine that attenuates the inflammatory processes induced by TNF-α, IL-6, and IL-1 while up-regulating the release of IL-1RA (14). IL-10 is negatively correlated with BMI, PFM, and fasting glucose levels. Low levels of IL-10 are associated with both the metabolic syndrome and type II diabetes **(Ballak et al., 2015)**. Tumor necrosis factor (TNF-α) is an adipokine, that promotes insulin resistance of obesity and non-insulin dependent diabetes mellitus (NIDDM). Multiple studies stated that in obese condition expression level of TNF- α is elevated **(Hotamisligil et al., 1994)**. In our study, TNF- α level is decreases after treatment with DHA extracted from *Polynemus paradiseus* oil.

Chapter 7: Conclusions

7. Conclusions:

'Polynemus paradiseus' is a common fish which largely available in sea coastal area of West Bengal region. It is locally available indigenous food which easily affordable for all community people. It has been demonstrated that the (n-3) fatty acid docosahexaenoic acid (DHA, C 22:6) found in fish oil reduces body fat and fat formation in mice. Recent years have seen a rise in interest in the effects of DHA on the metabolism of adipose tissue and their prevention of obesity. In this study we evaluated the presence of neutral lipids by Oil Red O staining. Oil Red O staining was used as an indicator of adipogenesis. Here, cells were treated with different doses (1.25, 2.5, 3.75 & 5 µM/ml) of DHA. DHA treatment during differentiation suppressed lipid accumulation, as shown by dose- dependent decreases droplet size and percentage of lipid area. Among the all doses we found 3.75 µM dose was more effective than other doses. Leptin was increased in 2.5 and 3.75 µM dose treatment than control by 1.06-fold. Expression level of adiponectin was increased by 2.16-fold and 1.65-fold, after treatment with 2.5 µM and 3.75 µM doses of DHA. The inflammatory marker, IL-6 was found decreased by 1.19-fold after treatment of 3.75 µM dose of DHA. We also measured the expression of peroxisome proliferator-activated receptor-α(PPAR-α) and FAS. Expression of PPAR-α level was increased 1.24-fold and FAS level was decreased by 1.12-fold after treatment of DHA. To further investigate the effects of dietary fatty acids on cytokine production by adipocytes, the effects of anti-inflammatory cytokine, IL-1ra, IL-10 and inflammatory cytokine TNF-α were assessed. Here, cells were treated with different doses (1.25, 2.5, 3.75 & 5 µM/ml) of DHA. Among the all doses, treatment with 3.75 µM/ml DHA shown more effective result. So based on these tests and parameter we can confirm that the extracted oil of '*Polynemus paradiseus*' containing DHA are helpful in treatment of obesity.

Chapter 8: Future Scope

8. Future Scope:

- Few drugs like orlistat, lorcaseion, liraglutide, phentermine- topiramate are available in the market but they are not free from side effects. They have lots of adverse effect to the body such as steatorrhea, dizziness, dry mouth, nausea, constipation, stomach pain, heart burn, headache, stomach ulcers etc.
- So, if we can cure obesity through diet, we must be minimizing the adverse effect of drugs and side by side have possibilities to minimize the risk of obesity related metabolic disorders also by inclusion of marine fish that contain much more amount of unsaturated fatty acids. On the basis of our study, we will develop a functional food by using *Polynemus paradiseus* which will help to reduce the risk of obesity and obesity associated metabolic disorders.

Chapter 9: References

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