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Analytical characterization and microbial studies of muscle lipid of Indian threadfin (*Polynemus indicus*) of the Bay of Bengal

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Abstract
Lipid was extracted from the muscle of Indian threadfin (*Polynemus indicus*) by solvent extraction method and then purified. Various physico-chemical properties of the lipid sample including cholesterol content were determined and compared with other standard fats and oils. Fatty acid composition of the lipid sample was investigated by Thin Layer Chromatographic (TLC) examination. Gas Liquid Chromatographic (GLC) examination was performed by the methyl esters mixture prepared from the lipid sample for the quantification of fatty acids. The lipid containing selected muscle was analyzed quantitatively for the determination of percentages of protein and mineral (N, P, K, Ca, and Na). The muscle was also analyzed for determining the amount of several metals by using Atomic Absorption Spectrophotometric method. Microbial activities (bacterial activity against nine bacteria and fungal activity against seven fungi) of the lipid were studied by standard procedures.

Keywords: *Polynemus indicus*, lipid, GLC, metal analysis, polyunsaturated fatty acid, fish nutrition

1. Introduction
The southern part of Bangladesh is completely covered by the Bay of Bengal rich in marine fishes and aquatic animals. Most of the people in Bangladesh have to rely heavily on fish as a source of animal protein and about 80% of the animal protein in our diet comes from fish only [1]. Indian threadfin, locally known as *Lakhua*, is an important part of the fishery resources of Bangladesh. It is also a valuable natural resource of the country. It is widely found in Indo-West Pacific, known from Pakistan to Papua New Guinea including India, Srilanka, Bangladesh, Myanmar, Thailand and Indo-Australia Archipelago. Currently, the biochemical effects of fish oils in human health and nutrition [2, 3] have placed renewed focus on the apparent difference in the compositions of fish oils [4]. It needs to be mentioned that fish lipids contain highly unsaturated fatty acids which reduce the cholesterol and LDL (low density lipoprotein) blamed for causing heart disease. The fish lipids also increase the level of HDL (high density lipoprotein) known as a friendly cholesterol for its role in cleaning LDL deposited in blood vessels [5, 6]. Indian threadfin may contain pharmaceutically important and physiologically active essential fatty acids (ω-3 and other PUFA's) in their body. In the current study, the lipid and fatty acids of the muscle of Indian threadfin (*Polynemus indicus*) was analyzed with a view to exploring the possibilities of commercial utilization of this highly available species as a source of marine oils and polyunsaturated fatty acids of ω-3 series. The lipid sample was also evaluated in terms of biological activities (bacterial and fungal activities) for pharmacological aspects. If proper measure is taken to analyze the muscle lipid of title fish it may have great industrial, pharmaceutical and nutritional uses. It was thus our objective to extract the lipid from the muscle of Indian threadfin and to investigate fatty acid pattern and properties.

2. Materials and Methods
The export quality title fish Indian threadfin (*Polynemus indicus*) was collected from the local fish market in marine city Chittagong which is a major coastal seaport (22°22′0″N 91°48′0″E) and financial centre in southeastern Bangladesh. The specimen was identified jointly in the Institute of Marine Sciences & Fisheries and Department of Zoology, University of Chittagong. After then the fish was preserved in deep freezer for a few days.

The selected Muscle was separated and preserved until extraction and further chemical investigation.

Oil extraction from the muscle of fish was carried out by using Bligh and Dyer method. Combined extract was recovered with a rotary evaporator at 45 °C. To obtain lipid this extract was dried under flushing with a slow stream of nitrogen gas for the removal of residual solvent. Analytical grade chemicals and reagents were used. Solutions were prepared according to the standard procedures [7-9]. The amount of total lipid was determined gravimetrically. Moisture content, fibre content, ash content and protein content of the de-oiled muscle of Indian threadfin were determined by standard methods [10]. A weight portion of the muscle of Indian threadfin was first dried in an oven at about 100-105 °C for 4-5 hours to remove moisture and then burnt into ash in a muffle furnace at about 600 °C for 4 hours [11]. The refractive index of the lipid sample was determined by Abbe refractometer (Model; DTM-1 Atago Co. Ltd). The specific gravity of the lipid sample was determined by Specific Gravity Bottle. The viscosity of the lipid sample was determined by Ostwald's viscometer at 30 ˚C.

Saponification value, saponification equivalent value, acid value and percentage of free fatty acid (as oleic acid), ester value, iodine value, acetyl value, peroxide value, thiocyanogen value, Titre value, Richert-Meissl value and Polenske value, Henher value, Kirschner value, Elaiden test result and quantity of unsaponifiable matter of the muscle lipid of Indian threadfin were determined by standard methods [12-16]. Thin layer chromatographic (TLC) investigation of the fatty acids present in the lipid sample was carried out in various solvent systems [17]. Fatty acid composition of lipid sample was quantified by gas-liquid chromatography (GLC) [18]. Cholesterol of the sample was determined with the standard method [19]. By applying the standard methods, percentages of minerals (N, P, K, Ca and Na) of lipid containing muscle were determined [20]. For analysis of metal content of muscle of the fish, the sample solution was prepared by using wet-digestion method and was stored in a refrigerator until analysis. An exactly similar blank solution of 100 ml was prepared for the test. For determining the presence and amount of metals in the fish sample, an Atomic Absorption flame emission spectrophotometer (Thermoscientific ICE 3000 series) was used [21, 22]. For the study of antibacterial activities, the disc diffusion method was followed. The antifungal activity was assessed by food poison technique [23].

Fig: Indian threadfin (*Polynemus indicus*)

3. Results and Discussion

3.1 Physical characteristics

The refractive index (RI) of the muscle lipid of Indian threadfin was found to be 1.4630 at 30 °C. The refractive power of oils or fats varies widely and chiefly governed by the proportion and degree of unsaturation present. It is a characteristic of various classes of fats or oils. The present result indicates that the muscle lipid from the specimen contained moderate amount of unsaturated fatty acids. The specific gravity of the lipid solution of the muscle lipid of Indian threadfin was found to be 0.925 at 30 °C. The viscosity of the lipid solution of the muscle lipid of Indian threadfin was found to be 333.001 milipoise at 30 °C. The energy of activation for viscous flow was also calculated and found to be 5.693 Kcal. From the result of this experiment, we got an idea about the intermolecular hydrogen bonding in the lipid sample. The present result as shown in Table 1 suggests that a few hydroxyl groups and few free acid molecules may present in the lipid sample.

Table 1: Physical constants of the lipid sample of Indian threadfin and other lipid samples

Name of the sample	Refractive index	Specific gravity	Viscosity (mp)
Muscle lipid of Cuttle fish	1.4731	0.9735	320.325
Brain lipid of Baghda Chingri	1.4736	0.941	303.260
Liver lipid of Blue Spotted Fantail ray	1.4760	0.9575	325.325
Muscle lipid of Indian threadfin	1.4630	0.925	333.001

The moisture content of the de-oiled muscle of Indian threadfin was found to be 0.07%. The moisture content in fixed oils or fats varies slightly and only small amount is generally present. The fiber content of de-oiled muscle of Indian threadfin was found to be 1.59%. The ash content of the muscle of Indian threadfin was found to be 0.33%. The protein content of de-oiled muscle lipid of Indian threadfin was found to be 4.3%.

3.2 Chemical properties of the lipid sample

The saponification value and saponification equivalent value of the muscle lipid of Indian threadfin was found to be 263.86 and 212.62 respectively (Table 2). The saponification value is inversely and the saponification equivalent value is directly proportional to the average molecular weight or chain length of the fatty acids present in the fat or oil. The results clearly indicate that the lipid sample contains higher proportion of high molecular weight fatty acids.

The acid value of muscle lipid of Indian threadfin was found to be 0.53 (Table 2). The percentage of free fatty acid (FFA), as oleic, was calculated from acid value and was found to be 0.27 (Table 2). Acid value indicates the proportions of free fatty acid in the oil or fat. The free fatty acid is produced by the hydrolytic decomposition of the oil or fat. Thus, low acid value is an indication of freshness of the oil or fat and suitability of the lipid for edible purpose.

The ester value of the muscle lipid of Indian threadfin was found to be 263.33 (Table 2). This value indicates the amount of ester present in the lipid sample.

Iodine value gives an estimation of the degree of unsaturation and so of the relative amounts of unsaturated fatty acids in the triglyceride molecules of the fat or oil. Iodine value of muscle lipid of Indian threadfin was found to be 68.15 (Table 2). The value indicates that the lipid sample contains lower to moderate proportion of unsaturated fatty acids and is of non-drying type.

The peroxide value is an indication of unsaturation present in fats and oils. The more unsaturated fats or oils absorb more oxygen, form greater amounts of unstable hydroperoxides and show higher peroxide values. The peroxide value of muscle lipid of Indian threadfin was found to be 39.29 (Table 2). It can be concluded from the result that the muscle lipid under investigation contained good amount of unsaturated fatty acids.

The acetyl value of the muscle lipid of Indian threadfin was found to be 10.97 (Table 2) which indicates low content of free hydroxyl groups may present in the lipid sample.

The thiocyanogen value of the muscle lipid of Indian threadfin was found to be 56.34 (Table 2). This observation is in conformity with the finding that the lipid sample has moderate iodine value and peroxide value.

The titre value of the muscle lipid of Indian threadfin was found to be 26.14 (Table 2). This value indicates that the lipid sample is of fat type which supports its semisolid condition at room temperature.

The Henher value of the muscle lipid of Indian threadfin was found to be 20.58 (Table 2). This result indicates the presence of higher percentage of water insoluble non-volatile fatty acids in the lipid sample.

The Unsaponifiable matter in the muscle lipid of Indian threadfin was found to be 0.71% (Table 2). Unsaponifiable matters are defined as those substances which are not saponified by alkali and which are soluble in ether or petroleum ether. In general, if a fixed oil or fat contains unsaponifiable matter in excess of about 2% there is reason to support adulteration. The result indicates that the lipid sample may contain a small amount of unsaponifiable matter such as sterols, vitamins A & D, hydrocarbons etc.

The Polenske value of the muscle lipid of Indian threadfin was found to be 12.63 (Table 2). The Polenske value represents a measure of volatile water insoluble but alcohol soluble fatty acids. The Polenske value as obtained is a support of the small amount volatile water insoluble but alcohol soluble fatty acids in the lipid sample.

The Reichert-Meissl (R-M) value of the lipid sample was found to be 3.81 (Table 2). The lower R-M value indicates the presence of low content of volatile water soluble fatty acids in the sample.

The Kirschner value of the muscle lipid of Indian threadfin was found to be 0.43 (Table 2). This indicates the presence of trace amount of fatty acids in the Reichert-Meissl distillate which forms soluble silver salt.

The muscle lipid of Indian threadfin was found to form cloudy solution with bromine and a precipitate appeared due to the insoluble bromide during the experiment. Thus, the lipid is a marine oil (fish oil).

The muscle lipid of Indian threadfin was found to form treacle-like consistency with mercuric nitrate, Hg $(NO_3)_2$ solution after 24 hours during the experiment. Thus, the lipid of Indian threadfin is of non-drying type and confirmed by iodine value.

The amount of cholesterol in the muscle lipid of Indian threadfin was found to be 14.66 mg/g. It can be suggested that the muscle lipid of Indian threadfin is more suitable for edible purpose.

The effect of storage time on the muscle lipid of Indian threadfin showed a significant variation in different values. Acid value, peroxide value increased with increasing time of storage and R-M value, thiocyanogen value, titre value and iodine value decreased with increasing time of storage. It means that the quality of the lipid deteriorated with increasing time of storage.

3.3 Chromatographic examination of muscle lipid of Indian threadfin

3.3.1 TLC Analysis

The muscle lipid of Indian threadfin was subjected to TLC examination and its fatty acid composition was identified by comparing the R_f values of different spots of chromatograms with those of standard fatty acids as reported (Table 3) earlier in different solvent systems. From the chromatogram three spots were identified as palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$) and oleic acid ($C_{18:1}$) in the muscle lipid of Indian threadfin.

Table 2: Chemical constants of muscle lipid of Indian threadfin and some related fats and oils

Name of the Sample	S.V.	S.E.V.	A.V.	F.F.A. (%) (as oleic)	E.V.	I.V.	P.O.V.	Acetyl Value (%)	T. V.	Titre value (°C)	H.V.	U.S.M. (%)	P.V.	R.M.V.	K.V.
Olive oil	190-195	287-295	0.6-1.5	0.25-0.60	---	80-88	---	10.4	75-83	17.26	0.6	0.5-1.2	0.5	0.6-1.5	---
Sunflower oil	190-194	287-295	0.6-2.4	0.15-0.45	---	125-140	---	---	78.4-81.3	17	---	0.3-0.9	---	0.5	---
Soyabean oil	190-195	287-295	1.2-1.5	0.35-0.85	---	129-137	---	---	77-85	22-27	---	0.7-1.6	0.2-1	0.5-2.55	---
Coconut oil	255-260	210-250	2.5-10.0	---	---	8.2-9.6	---	---	6.1-7.0	20-24	82	0.15-0.7	15-17	7.8-8.0	---
Palm-Kernel oil	248	220-250	220-250	---	---	15-18	---	---	---	---	94.2	---	---	28	---
Sardine oil	189.8-193.8	---	2.2-21.7	---	---	138-177	---	---	---	---	---	---	---	---	---
Whale oil	184-200	---	0.3-51	---	---	126.9	---	---	---	---	---	---	---	---	---
Muscle lipid of	203.25	276.01	3.108	1.56	---	92.55	55.05	10.255	52.54	---	93.27	0.74	0.764	0.965	---

Hilsa															
Muscle lipid of Flathead mullet	182.5	307.397	2.83	1.26	---	125.92	---	11.22	68.09	26.60	79	1.16	0.36	0.92	---
Muscle lipid of Cuttle fish	260.87	215.05	1.78	0.89	258.77	106.82	109.45	12.95	54.82	27.5	77.98	1.10	0.72	0.91	0.424
Muscle lipid of Indian threadfin	263.86	212.62	0.53	0.27	263.33	68.15	39.29	10.97	56.34	26.14	20.58	0.71	12.63	3.81	0.43

Abbreviations: S.V.= Saponification Value; S.E.V.= Saponification Equivalent Value; A.V.= Acid Value; F.F.A.= Free fatty acid; E.V.= Ester Value; I.V.= Iodine Value; P.O.V.= Peroxide Value; T.V.= Thiocyanogen Value; H.V.= Henher Value; U.S.M.= Unsaponifiable Matter; P.V.= Polenske Value; R.M.V.= Reichert-Meissl Value; K.V.= Kirschner Value. "---" = Data not available.

Table 3: The R_f values (most related) of thin layer chromatographic examination of the muscle lipid of Indian threadfin

Solvent System	R_f values of standard fatty acids					R_f values obtained from the spots of lipid sample			
	PA	SA	OA	LA	EA				
P:E:A (70:30:1)	0.942	0.961	0.417	---	---	0.691	0.846	**0.943**	---
P:E (80:20)	0.941	0.943	0.287	0.933	0.361	0.285	0.662	0.758	0.846
P:H (80:20)	0.813	0.832	0.316	---	---	0.317	0.769	0.923	---
H:E (80:20)	0.815	0.821	0.201	0.641	0.201	0.822	---	---	---

PA- Palmitic Acid, SA-Stearic Acid, OA- Oleic Acid, LA-Linoleic Acid, EA- Erucic Acid P:E:A = Petroleum ether: Ether: Acetic Acid; P:E = Petroleum ether: Ether; P:H = Petroleum ether: Hexane; H:E = Hexane: Ether

3.3.2 GLC Analysis

The chromatographic method especially gas-liquid chromatography (GLC) radically changed the possibilities for obtaining compositional data from biological material. The continuous increase in the refinement and exactness of the chromatographic separations has made them a powerful analytical tool. Their simplicity together with their rapidness has resulted in the availability of extensive compositional data on the fatty acid composition of lipids. Identification and quantification of fatty acids in the lipid sample was furnished by GLC and the data shown in Table 4.

Table 4: Fatty acid composition of the methyl ester mixture derived from the lipid of Indian threadfin by GLC

Sl No	Name of fatty acid	Relative percentage (%)	Sl No	Name of fatty acid	Relative percentage (%)
1	Lauric Acid (C12:0)	---	15	Linolenic Acid (C18:3)	0.7188
2	Myristic Acid (C14:0)	5.0514	16	Arachidic Acid (C20:0)	0.2072
3	Myristoleic Acid (C14:1)	0.9698	17	Eicosenoic Acid (C20:1)	0.9591
4	Pentadecyclic Acid (C15:0)	0.7325	18	Eicosadienoic Acid (C20:2)	0.1826
5	Palmitic Acid (C16:0)	36.3674	19	Eicosatrienoic Acid (C20:3)	0.1060
6	Palmitoleic Acid (C16:1)	13.2323	20	Arachidonic Acid (C20:4)	2.2254
7	Hexadecadienoic Acid (C16:2)	0.9654	21	Eicosapentaenoic Acid (EPA) (C20:5)	2.4392
8	Hexadecatrienoic Acid (HTA) (C16:3)	0.9930	22	Behenic Acid (C22:0)	0.1915
9	Margaric Acid (C17:0)	0.5088	23	Erucic Acid (C22:1)	---
10	Heptadecanoic Acid (C17:1)	0.0031	24	Docosadienoic Acid (C22:2)	0.5638
11	Stearic Acid (C18:0)	9.1509	25	Docosatetraenoic Acid (C22:3)	0.3740
12	Oleic Acid (C18:1)	14.5988	26	Adrenic Acid (C22:4)	1.1411
13	Vaccenic Acid (C18:1)	5.9001	27	Docosapentaenoic Acid (DPA) (C22:5)	1.5901
14	Linoleic Acid (C18:2)	0.7805	28	Docosahexaenoic Acid (DHA) (C22:6)	0.0472

3.4 Estimation of minerals

Most of the people of our country have been suffering from protein malnutrition. From the Table 5, it is evident that Indian threadfin contained good amount of nitrogen (0.688%) as well as protein (proteineous nitrogen) which are well balanced in respect of essential amino acids. The percentage of phosphorus in the muscle of Indian threadfin was 0.495. The result indicates that phospholipids may present in the lipid which was extracted from the muscle of fish sample. The percentage of potassium in the muscle of Indian threadfin was found to be 0.041. Consumption of this marine species may be helpful to increase blood pressure for those people having low blood pressure. So, this may be a remedy of low blood pressure. The percentage of sodium in the muscle of Indian threadfin was found to be 0.35. The percentage of calcium in the muscle of Indian threadfin was found to be 0.010. Above all, maximum consumption of Indian threadfin should be encouraged for their ready supply to different remote regions under careful processing to avoid putrefaction.

Table 5: Percent of N, P, K, Na and Ca in muscle of Indian threadfin

Name of the sample	N (%)	P (%)	K (%)	Na (%)	Ca (%)
Muscle lipid of Cuttle fish	6.533	1.2374	1.118	---	0.798
Brain lipid of Baghda Chingri	3.540	0.7262	1.123	---	0.914
Liver lipid of Blue Spotted Fantail ray	4.099	2.7500	1.180	---	0.614
Muscle lipid of Indian threadfin	0.688	0.4950	0.041	0.35	0.010

'---' indicates data not available.

3.5 Metal analysis of muscle of Indian threadfin

Determination of several metals were performed for the selected muscle of the fish sample. Among the metals found in the muscle (as shown in Table 6), the Magnesium (Mg)

content was found to be 123.05 ppm. The level is far below the limit for fish proposed by World Health Organization (WHO) and safe within the limits for human consumption in the edible part of the studied fish. The Chromium (Cr) of muscle of the fish sample was 12.13 ppm. The Lead (Pb) of muscle of the fish sample was 1.55 ppm. It is to be mentioned here that Cobalt (Co), Cadmium (Cd), and Nickel (Ni) were not found in the muscle of the fish sample. Iron (12.14), copper (3.40) and zinc (6.82) were also found in good amount. The Manganese (Mn) content of muscle of the fish sample was 0.63 ppm. The Arsenic (As) content of Indian threadfin was 0.40 ppm. It can be concluded from the finding that the amount of As is not above the danger level. Hence, the people can easily take or eat the fish without any fear or hesitation of As poisoning.

Table 6: Concentration of different metals in muscle of Indian threadfin

Metals	Concentration (ppm)	Metals	Concentration (ppm)
Fe	12.14	Ni	-
Co	-	Pb	1.55
Cu	3.40	Mn	0.63
Cr	12.13	As	0.40
Zn	6.82	Mg	123.05
Cd	-		

"-" means that the metal was not found in muscle of Indian threadfin.

3.6 Microbial activities of the lipid sample

In the present study, the muscle lipid of Indian threadfin was selected and screened for antibacterial activities against nine bacteria and antifungal activities against seven fungi.

3.6.1 Antibacterial test

To perform the antibacterial activities of the lipid sample, it was subjected to antibiosis test against several bacteria such as *Salmonella sp*., *Shigella sp*., *Lactobacillus casei, Proteus vulgaris*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Bacillus cereus*, *Pseudomonas sp.* For the test, chloroform was used as a solvent to prepare desired solution (10%) of the lipid sample and proper control was maintained. The lipid sample did not exhibit antibacterial activity against any of the bacteria tested under the specified conditions. So, it may be said that the sample does not have any activity against the above mentioned bacteria.

3.6.2 Antifungal activities

The antifungal activities of the lipid sample were studied against seven phyto-pathogenic fungi. The muscle lipid of Indian threadfin showed lower zone of inhibition on mycelial growth of *Fusarium equiseti, Aspergillus fumigatus*, *Alternaria alternata*, *Curvularia lunata, Collectortrichum sp*., *Asperigillus flavus* and *Asperigillus ochraceus* respectively.

4. Conclusion

In this study physico-chemical characterization and microbial studies of muscle lipid of Indian threadfin (*Polynemus indicus*) was performed. The presence of moderate amount of unsaturated fatty acids in the lipid sample was confirmed by R.I., S.V., I.V. and T.V. Low content of volatile water-soluble and volatile water-insoluble fatty acids was established by R.M.V. and P.V. Low free hydroxyl group content was confirmed by the acetyl value of the lipid sample. Percentage of F.F.A. validated the suitability of the oil for edible purpose. Nondrying nature of the muscle lipid of Indian threadfin was pointed out by I.V. and confirmed by Elaiden test. Chromatographic examinations (TLC and GLC) substantiated the presence of some important fatty acids in the lipid sample. It can be said that the levels of metals in the fish sample are below the limit for fish proposed by World Health Organization and safe within the limits for human consumption in the edible part of the studied fish. However, antimicrobial screening results could not provide us with any decisive conclusion.

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