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ISOLATION AND CHARACTERIZATION OF A NOVEL ANTICANCER MUSCLE PROTEIN FROM EDIBLE MARINE CATFISH *TACHYSURUS DUSSUMEIRI*

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Keywords:

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ABSTRACT: Cancer, the uncontrolled growth of cells, has been estimated as a major cause of death among human population. Although there are many potential drugs, a much effective bioactive drug with less side effects are still in its pioneer stage of research. The aim of the study was to isolate, purify and characterize the anticancer muscle protein from edible marine catfish, *Tachysaurus dussumieri*, so as to project it as an alternative medicine for cancer. The anticancer muscle protein was purified to its homogeneity from the edible marine catfish *Tachysurus dussumieri* through ammonium sulphate fractionation and ion exchange chromatography. Both partially purified crude fractions and isolated purified muscle protein were screened for its anticancer activity by MTT assay. The fraction III revealed prominent anti-cancer activity which was subjected to MALDI-TOF MS analysis for its molecular weight confirmation. Further, Peptide Mass Finger printing analysis (PMF) was executed to reveal its nature. To reconfirm its anticancer action, DAPI, PI staining and Comet assay were executed. The purified dimer having 13.285 kDa weight was homologous to Tumor Necrosis Factor (TNF)-3a. The Purified protein showed a remarkable inhibitory effect on the proliferation of human colon adenocarcinoma cell line (HT 29) at a concentration of 20 µg/ml. Comet assay and fluorescence microscopic analysis also confirmed the significant increase in DNA fragmentation and cellular degradation of HT 29 cells, when treated with isolated protein. These results indicated the potentiality of the protein as a colorectal cancer therapeutic agent.

INTRODUCTION: Cancer death worldwide is projected to rise continuously with an estimated 11.5 million deaths in 2030 and about 1,660,290 new cancer cases every year [1].

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Among the different types, colorectal cancer is found to hold third and second position in common cancers among men and women respectively [2]. Epidemiological studies have demonstrated that the development of colon cancer is closely associated with dietary habits and lifestyle [3].

Chemotherapy is one of the early approach for the treatment for cancer and was introduced into the worldwide clinics more than fifty years ago. Although Chemotherapy has been successful for the treatment of few cancer types such as testicular

cancer and certain leukemias, its potential for the treatment of common epithelial tumors of the breast, colon, and lung did not register much impact [4]. The new generation of chemotherapeutics affects the signals that promote or regulate the cell cycle, growth factors and its receptors, signal transduction pathways and pathways affecting DNA repair and apoptosis. However, commercially available chemotherapeutic drugs do produce a lot of side effects, even making it unaffordable by the common people. So the need of the hour is to develop high potential low cost anticancer agents from natural resources. Peptides produced by enzymatic hydrolysis of marine proteins were the alternative source of bioactive compounds with anticancer potential, since they have shown to have antioxidant and anti-proliferative properties. During 2005 and 2006, the antitumor and cytotoxic properties of 136 marine natural products were identified, many of which are novel compounds that belong to diverse structural classes, including terpenes and peptides. However, tissues of fish also constitute potential anticancer molecules for example, the squalamine, an aminosterol isolated from *Squalus acanthias* liver, was demonstrated to be a potent inhibitor of tumor growth and angiogenesis in several animal models [5].

TNF (Tumor Necrosis Factor) is well known cytokine; because of its ability to kill tumor cells *in vitro*. In fish, TNF has been cloned in rainbow trout [6] common carp and catfish [7]. Two copies of TNF have been cloned in rainbow trout and carp, which have been named TNF-1a and TNF-2a and were reported to possess apoptotic property [8]. Apoptosis is a remarkable feature of drug induced cell death of the proliferating cancer cells. The overall contribution of apoptotic defects to clinical multidrug resistance remains under debate [9]. Treatment of cancer cells (A549) with the crude and partially purified epidermal mucus protein from marine catfish *T. dussumieri* was reported to cause a dose – dependent decrease in cell viability, structural damage and mitochondrial dysfunction [10]. However, scarce literature is available till date for marine anticancer agents from catfishes. Hence, in the present scenario, the isolation and characterization of protein with anticancer potential has got a great significance in chemotherapeutic drug development [11]. Thus, in this investigation, an attempt was made to isolate and characterize a bioactive protein from the muscle of the marine catfish, *Tachysaurus dussumieri* with potential anticancer activity against human cancer cell line.

MATERIALS AND METHODS:

Chemicals and Cell Lines: Human adenocarcinoma colon (HT 29) and laryngeal (Hep2) cancer cell lines were obtained from National Centre for Cell Science (NCCS), Pune, India. Ammonium sulphate, Tris, NaCl, KCl, DEAE Cellulose, Standard protein molecular weight marker, Coomassie Brilliant Blue (R-250), Dulbecco's Modified Eagle Medium (DMEM), Trypsin-EDTA, Fetal Bovine Serum (FBS), 3-(4, 5- Dimethyl thiazol-2yl)-2, 5-dimethyltetrazolium bromide (MTT), Propidium Iodide (PI), 2-4', 6-diamidino-2-phenylindole (DAPI), Dimethyl sulphoxide (DMSO), Sodium bicarbonate and antibiotic solution were purchased from Hi-Media laboratories Ltd., Mumbai, India.

Collection of Fish: The cat fish *T. dussumieri* were collected from Kovalam coast, Chennai, Tamil Nadu, India (Latitude: 12º47'33''N; Longitude: 80º15'10.8"E) and identified by a Zoologist in Department of Zoology, University of Madras, Guindy Campus, Chennai.

The catfish muscle collected immediately, washed with Milli Q water followed by Tris buffer and stored at −20ºC until further used.

Extraction of Protein: The extraction of crude protein from the fish muscle was performed by the method of Arulvasu *et al*., 10. Briefly, the muscle tissue (100 g) were minced using homogenate medium (HM) having 0.013 M Tris, 0.12 M NaCl and 0.003 M KCl at pH 7.4, in the ratio of 1:3. The homogenate was filtered and centrifuged (8,000 × g; 15 min at 4 ºC). The cell free supernatant was collected, subjected to ammonium sulphate precipitation at 20 (Fraction I), 50 (Fraction II) and 100% (Fraction III) saturation in a magnetic stirrer for 4 -5 h at 4 ºC. Each fraction of precipitated proteins were collected by centrifugation at 7000 × g for 15 min at 4 ºC. The precipitate was dissolved in minimum volume of HM and dialysed against the low strength of HM using a membrane (12 kDa cut off) and lyophilized.

Quantification of Protein and PAGE Analysis: Total protein content of the fractions were

quantified by following the method of Bradford 12 using BSA (fraction V) as a standard. The protein profile of the lyophilized fractions (1 mg/ml) were analyzed using Native Polyacrylamide Gel Electrophoresis (Native-PAGE) and Sodium Dodecyl Sulphate - Polyacrylamide Gel electrophoresis (SDS-PAGE) as described by Davis 13 and Laemmli 14 respectively.

Purification and Characterization of Bioactive Protein:

DEAE Cellulose Column Chromatography: A bioactive protein from fraction III of 100% ammonium sulphate saturation was further purified using DEAE cellulose anion exchange chromatography as described by Veeruraj *et al*., [15] with some slight modifications. The DEAE cellulose was packed in a glass column (8 × 3 cm) and washed with in sterile Milli Q water, followed by 4 M NaCl. The column was equilibrated using 5 bed volumes of binding buffer (1 M Tris-HCl; pH 7.4) at 4 ºC. The Fraction III was loaded onto the DEAE column and incubated for 1 h. To remove the unbound proteins, the column was washed with 5 bed volumes of 20 mM Tris-HCl, pH 7.4. Bound proteins were eluted by linear gradient of NaCl (0-1 M) in binding buffer at a flow rate of 0.5 ml/min. All chromatographic fractions (2 ml) were monitored at 280 nm. The purified fraction was dialyzed and lyophilized. Homogeneity of eluted fractions were analysed by Native and SDS-PAGE.

SDS-PAGE: Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by following the method of Laemmli 14 using 10% acrylamide gel. After electrophoresis, the gel was stained with CBB R-250 followed by de-staining (methanol: acetic acid: water (4:1:5).

Molecular Weight Determination by MALDI TOF: The molecular weight of the purified protein was determined using MALDI-TOF MS (Bruker Daltronics, Bremen, Germany).

Peptide Mass Finger Printing Analysis MALDI – TOF: Peptide Mass Finger printing analysis of bioactive protein from *T. dussumieri* was carried out as described in Radhakrishnan *et al*., [16]. Gel bands were manually excised and subjected to in-gel chemical modification of cysteine residues with dithiothreitol and iodoacetamide, followed by tryptic digestion and peptide extraction. Concentrated peptides were spotted (0.5 µl) on a matrix assisted laser desorption/ionization (MALDI) plate, followed by 0.5 µl α-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile + 0.1% trifluoroacetic acid. MALDI peptide mass spectra were calibrated using matrix ion peaks as per international standard. The data obtained under mass spectrum were subjected for data base (NCBI non-redundant / SwissProt) searching using the program MASCOT (http:/matrixscience.com) analysis allowing up to one missed trypsin cleavage and monoisotopic mass tolerance of 1.2 Da. The calibrated peptide masses were searched with 200 ppm mass accuracy.

Cytotoxicity Assay: The cytotoxic effect of purified protein was tested by MTT method 17. Briefly, human cancer cell line HT 29 (1×10^5 cells/well) were plated in 96 well plate with DMEM containing 10% FBS. The cells were incubated for 24 h under 5% CO_2, 95% O_2 at 37 °C to allow the cells to attach to the bottom of the well. Then, the DMEM was removed from the cell culture plate, the cells were washed with PBS and treated with medium containing different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of Fraction I, Fraction II and fraction III of protein. After 24 h of incubation, MTT (3-(4,5-dimethythiazol- 2-yl)- 2,5-diphenyl tetrazolium bromide) (5 mg/ml; 10 µl) solution was added to each well and the cells were further incubated for 4 h at 37 ºC, 100 µl of DMSO was added and the crystals formed were dissolved by gentle resuspension. The same procedure was followed with the purified protein at concentrations of 20, 40, 60, 80 and 100 µg/ml. A micro plate reader was used to measure absorbance at 570 nm for each well. Growth inhibition rate was calculated as follows.

Percentage of growth inhibition = A570 of treated cells/A570 of control cells × 100

Morphological Examination: The cytomorphology of the HT 29 and Hep2 cells treated with partially purified fractions and purified protein was analysed as described by Arulvasu *et al*., [18] The cells were seeded at 1×10^5 cells/well into a six well plate containing coverslip and incubated overnight with DMEM.

Later, the old medium was replaced with fresh medium containing IC_{50} (0.6 mg/ml) and maximum concentration (1 mg/ml) of protein fractions and incubated for 24 h. The cytomorphology was observed using inverted phase contrast microscope (RTC-7A-RADICAL, India) and photographed. The same procedure was followed with the purified protein at a concentration of 20 and 100 µg/ml.

Propidium Iodide Staining: A purified protein was analyzed for its ability to induce cellular degradation of cancer cells by Propidium iodide staining as described by Arulvasu *et al*., [18] Briefly, cells were plated at 5×10^4 cells/well onto a six well plate containing cover slip. At >90% confluence, the cells were treated with 20 and 100 µg/ml of purified protein and a separate control was maintained. After 24 h incubation, cells were washed with PBS (2X) and fixed in methanol: acetic acid (3:1) for 10 min. After the fixation, cells were stained with 20 µl of PI (0.5 mg/ml) for 20 min. Then the nuclear morphology of cells were examined under inverted fluorescence microscope (RTC-7A-RADICAL, India).

DAPI Nuclei Staining: The DAPI staining method was performed following the method of Chen *et al*., 19 with slight modifications. After treatment, the cells were collected by centrifugation of 1500 rpm for 10 min at 37 °C and washed once with ice-cold phosphate-buffered saline (2X) and fixed in methanol: acetic acid (3:1) solution for 30 min. Then, fixed cells were placed on slides and stained with 20 µl of DAPI (1mg/ml) for 15 min. After DAPI staining, cells were visualized under inverted fluorescence microscope (RTC-7A-RADICAL, India).

Comet Assay: To study the DNA damage induced by purified protein, the Comet Single Cell Gel Electrophoresis (SCGE) neutral assay was performed following the method of Prabhu *et al*., [20]. The images of comets were used to estimate the individual nuclei DNA content of the damaged cells at 20 and 100 µg/ml concentration. The degree of DNA damage representing the total DNA fraction in the tail was evaluated. The amount of DNA damage in different sample was imaged and analyzed by CASP Lab software. The individual comet was studied to determine the percentage of tail DNA in the sample.

Statistical Analysis: Statistical analyses were performed using the SPSS commercial statistical package (version 16.0) and the significant means were compared using the Duncan test.

RESULTS:

Quantification and Profiling of Proteins: The protein from the muscle of marine catfish was extracted using Tris-HCl and precipitated with ammonium sulphate with 20, 50 and 100% saturation. The total protein content of the homogenate, dialyzed fractions I, II and III were found to be 130, 19.20, 144.06 and 180 mg/ml respectively **Table 1**. The protein profile for lyophilized fraction I, II and III was analyzed by 10% native and SDS-PAGE which revealed distinct protein of molecular weight ranging from 6.5 to 205 kDa **Fig. 1**.

TABLE 1: PURIFICATION OF BIOACTIVE PROTEIN FROM MUSCLE OF CAT FISH

S. no.	Fractions	Total protein (mg)
1	Homogenate	130.00±0.001
2	Fraction I	019.20±0.003
3	Fraction II	144.06±0.009
4	Fraction III	180.00±0.006
5	DEAE Cellulose	030.80±0.001

Values expressed as Mean ± SD of three replicates.

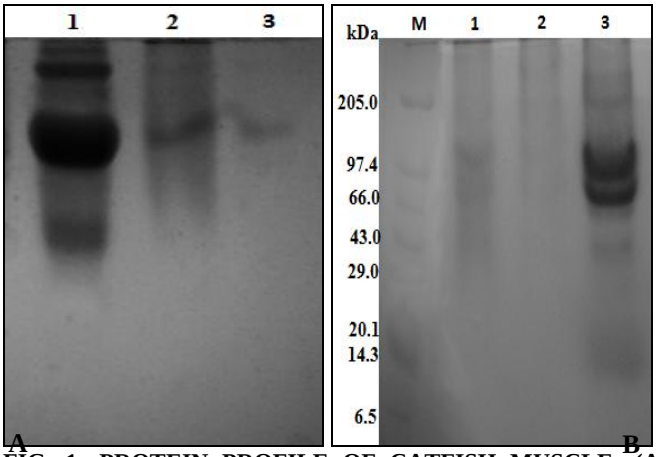


FIG. 1: PROTEIN PROFILE OF CATFISH MUSCLE; (A) NATIVE PAGE; LANE 1: FRACTION III; LANE 2: FRACTION II; LANE 3: FRACTION I AND (B) SDS-PAGE; LANE M: MOLECULAR WEIGHT MARKER (KDA); LANE 1: FRACTION I; LANE 2: FRACTION II; LANE 3: FRACTION III

Preliminary Screening of Anticancer Properties: The preliminary screening of ammonium sulphate precipitated fractions for anticancer potential was performed using HT 29 and Hep2 cancer cell lines by MTT assay. These results showed that the partially purified fraction III resulted in decreased

cell viability in both the cell lines tested **Fig. 2a** and **b**. But fraction III showed prominent anticancer activity against HT 29 cells at lower IC_{50} value of 0.6 mg/ml, compared to Hep2 cells which showed similar activity at 1 mg/ml.

After 24 h of incubation, destruction of monolayer with cell shrinkage forming spherical bodies was observed in both HT 29 and Hep2 cells compared to control **Fig. 3**.

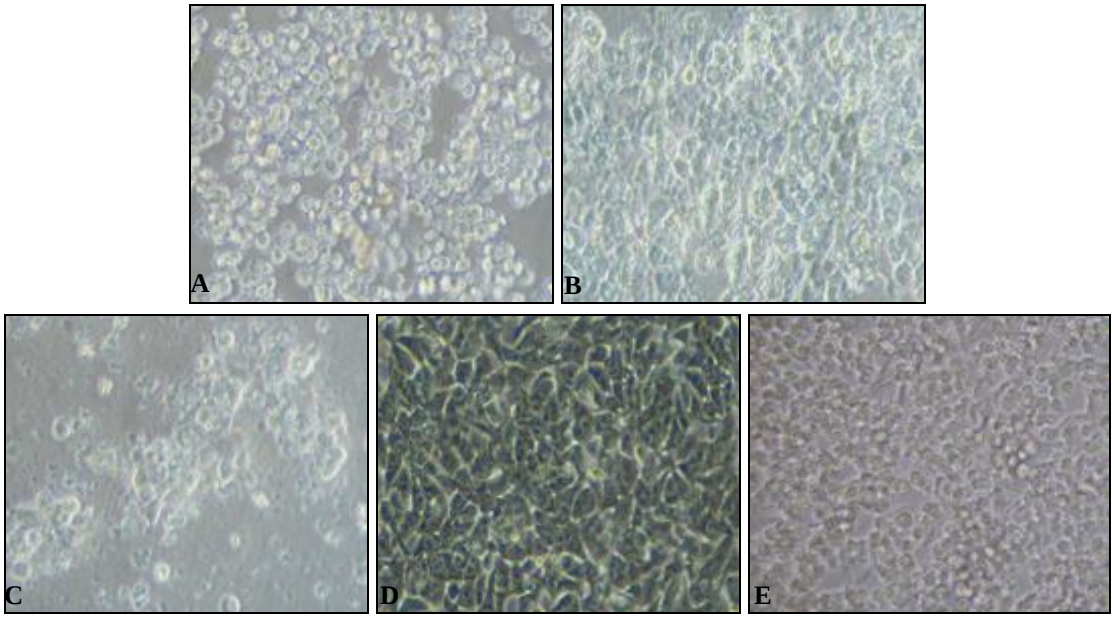


FIG. 2: MORPHOLOGY OF HT 29 AND HEP2 CELLS TREATED WITH BIOACTIVE PROTEIN FRACTION III OF CATFISH MUSCLE AFTER 24 h INCUBATION (20X MAGNIFICATION); (A) HT 29 CONTROL (B) HT 29 CELLS TREATED WITH 0.6 mg/ml, (C) HT 29 CELLS TREATED WITH 1 mg/ml, (D) HEP2 CONTROL (E) HEP2 CELLS TREATED WITH 1 mg/ml

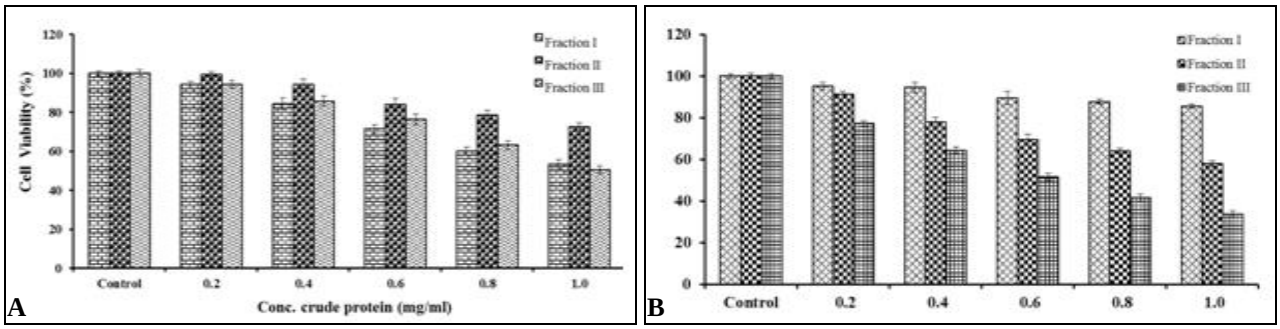


FIG. 3: EFFECT OF CRUDE MUSCLE PROTEIN FRACTIONS OF CATFISH ON THE VIABILITY OF CANCER CELLS AFTER 24 h INCUBATION; (A) HT 29 AND (B) HEP2. EACH POINT REPRESENTS THE MEAN ± S.D. OF 5 INDEPENDENT EXPERIMENTS. *p < 0.05 AND **p < 0.001 INDICATE SIGNIFICANT DIFFERENCES BETWEEN THE EXPERIMENTAL AND CONTROL VALUES

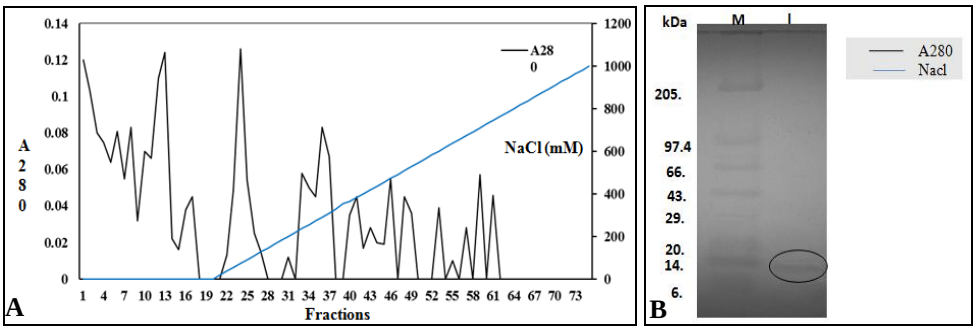


FIG. 4: (A) ELUTION PROFILE OF PROTEIN FRACTION III OF CATFISH USING DEAE CELLULOSE COLUMN CHROMATOGRAPHY AND (B) ANALYSIS OF PURIFIED BIOACTIVE PROTEIN FROM CATFISH MUSCLE; LANE M: MOLECULAR WEIGHT MARKER (KDA); LANE I: PURIFIED PROTEIN (SDS-PAGE)

Ion Exchange Chromatography: The bioactive protein was purified to homogeneity using DEAE cellulose column, elutions were monitored at 280 nm spectrophotometrically and a chromatogram was constructed **Fig. 4a**. All the fractions were subjected to SDS-PAGE. An active fraction was migrated as a single band on SDS-PAGE with a molecular weight of 14 kDa under reducing conditions and yielded 3% (30.80 mg/ml) of the total proteins present in homogenate **Fig. 4b** and **Table 1**.

Characterization of Protein: The homogeneity and exact molecular mass of isolated protein was further confirmed as 13.285 kDa **Fig. 5a** as evidenced by MALDI-TOF MS. It also revealed that the isolated protein is of dimeric nature. Peptide mass fingerprint (PMF) analysis and tandem mass spectrometric experiments were used to identification of proteins in fish muscle tissue. A similarity search in the NCBI non-redundant protein sequence database revealed that purified protein failed to exhibit 100% homology with any other reported animal peptides. However, Swissprot analysis of purified protein from muscles of *T. dussumieri* showed highest percentage of homology with Tumor Necrosis Factor-3 alpha from *Cyprinus carpio* and lowest homology with MHC class II beta chain from *Rutilus rutilus* **Fig. 5b**. Moreover, three putative proteins, with unknown function, from *Tetraodon nigroviridis* also showed homology with the isolated 13.285 kDa protein. This protein showed 7 identical peptides (TFFGVFAL, DNLSKENVTSK, QNQDGAFVSGGLK, VETD-NAKTFFGVFAL, MMDLESQPLPQEMVSR, NNIDWKQNQDGAFVSGGLK and AARSACV-HASNTEDVWY DTIYLGAAFSLR) of TNF-3a in the MASCOT homology search using trypsin digested partial homology BLAST **Table 2**.

TABLE 2: PEPTIDE MASS FINGERPRINTING (PMF) ANALYSIS OF MUSCLE BIOACTIVE PROTEIN OF CATFISH USING SWISSPROT

Swiss prot ID	Functional category	Molecular weight (kDa)	Peptides Matched	Start and end position	Peptide sequence of matched fragment
Q7T2Q3_CYPCA	Tumor necrosis factor-3 alpha from *Cyprinus carpio.*	2.5553	7	220-227	TFFGVFAL
				65-75	DNLSKENVTSK
				97-109	QNQDGAFVSGGLK
				213-227	VETDNAKTFFGVFAL
				2-16	MMDLESQPLPQEMVSR
				91-109	NNIDWKQNQDGAFVSGGLK
				168-196	AARSACVHASNTEDVWYDTIYLGAAFSLR
Q4SU67_TETNG	Putative protein from *Tetraodon nigroviridis*	6.4976	10	36-44	ESSWTGPPRD
				58-66	SKPSSFNDR
				518-526	SKSLYTSFR
				258-266	LVDMMERGK
				350-358	VVWVEELDK
				550-561	TGRNPGGHGGNR
				372-382	DSLTLVFVETK
				308-318	QTMMFSATFPK
				395-406	EGYACTSIHGDR
				247-264	GCHLLVATPGRLVDMMER
Q4S9M9_TETNG	Putative protein from *Tetraodon nigroviridis*	1.1343	5	93-97	AFEMS
				28-39	AHLANHDDLISK
				9-20	TFQAYLDSCHRR
				7-19	AKTFQAYLDSCHR
				48-65	AYLFNSVVNVGCGPAEER
CAG11485	Putative protein from *Tetraodon nigroviridis*	6.6958	8	5-14	QMAASNGLCR
				115-123	CMNTYGSYK
				568-579	MGEMAVDDIALR
				2-14	WPRQMAASNGLCR
				528-543	QYSPAVWGRTGGNGWR
				280-294	IPPNHHNHIPDRYEK
				158-176	GEVRCQCPSPGLQLAADGR
				115-142	CMNTYGSYKCYCLNGYMLLPDGTCGNAR
Q5W5J7_RUTRU	MHC class II beta chain from *Rutilus rutilus*	1.1410	4	60-70	HPAVLMCSAYR
				1-12	ELGVHNAESWNK
				17-29	IQQMTAQLEGYCK
				86-101	VVKSDVTSTEEMPNGD

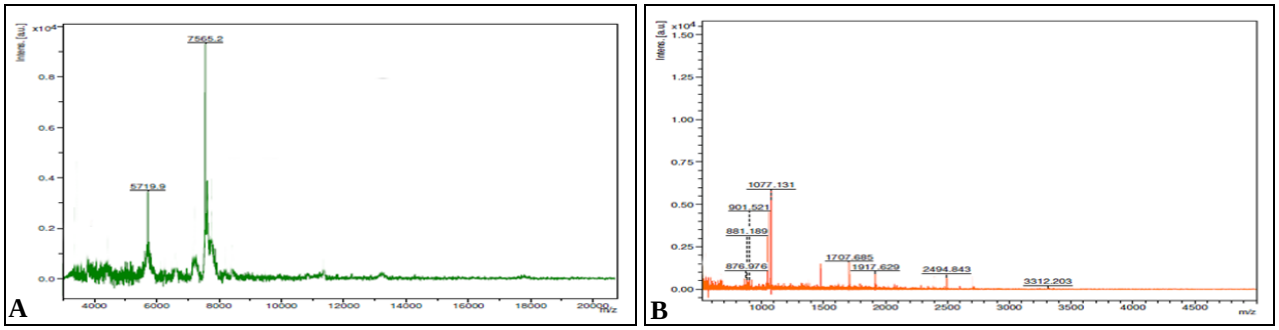
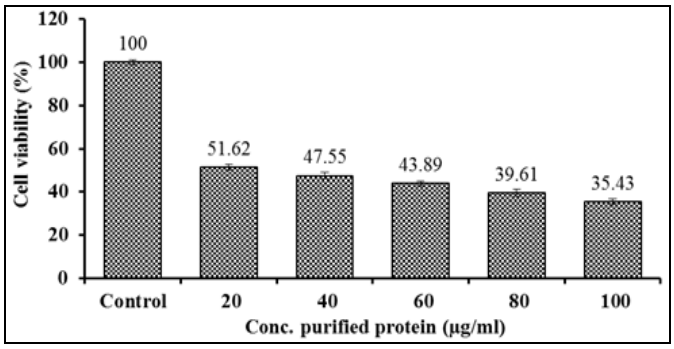


FIG. 5: MALDI-TOF MS ANALYSIS OF PURIFIED PROTEIN OF CATFISH MUSCLE; (A) MOLECULAR WEIGHT SPECTRUM AND (B) PEPTIDE MASS FINGERPRINTING SPECTRUM

Screening of Anticancer Activity of Purified Protein: The dose dependent inhibitory effect was observed in isolated protein from *T. dussumieri* treated HT 29 cells at different concentration, 20, 40, 60, 80 and 100 µg/ml. The IC_{50} value was obtained at 20 µg/ml after 24 h incubation **Fig. 6a**. In addition, exposure of the isolated protein on viable HT 29 cells resulted in formation of irregular confluent aggregates with round and polygonal cells **Fig. 6b**. Then, the nuclear morphology changes was analyzed using PI and DAPI staining with bioactive protein treated cells, which showed characteristic apoptotic changes such as chromatin condensation, or fragmentation of the nuclei after 24 h incubation **Fig. 7a-f**.



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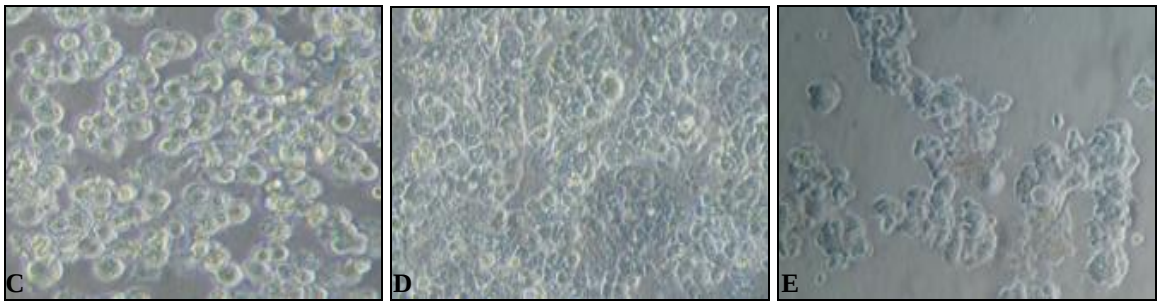

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FIG. 6: (A) EFFECT OF THE PURIFIED BIOACTIVE PROTEIN ON THE VIABILITY OF HT 29 CELLS AFTER 24 H INCUBATION (P < 0.001* INDICATE SIGNIFICANT DIFFERENCES BETWEEN THE EXPERIMENTAL AND CONTROL VALUES) AND (B) MORPHOLOGICAL CHANGES ON HT 29 CELLS TREATED WITH BIOACTIVE PROTEIN FROM CATFISH MUSCLE AFTER 24 h INCUBATION (20X MAGNIFICATION); (C) CONTROL D) 20 µg/ml, (E) 100 µg/ml TREATED CELLS.**

Further the DNA damage was confirmed by comet assay. Comets originating from damaged cells showed a distinct head with a tail. The damaged DNA tails of the treated cells were compared with appropriate control. The tail DNA (%) was found to be increased consecutively for control, cells treated with 20 and 100 µg/ml of bioactive protein whereas, the head DNA (%) was decreased sequentially for the same **Fig. 7g-i**. Maximum percentage of tail DNA % (35.22) was exhibited by the cells treated with 100 µg/ml concentration of bioactive protein.

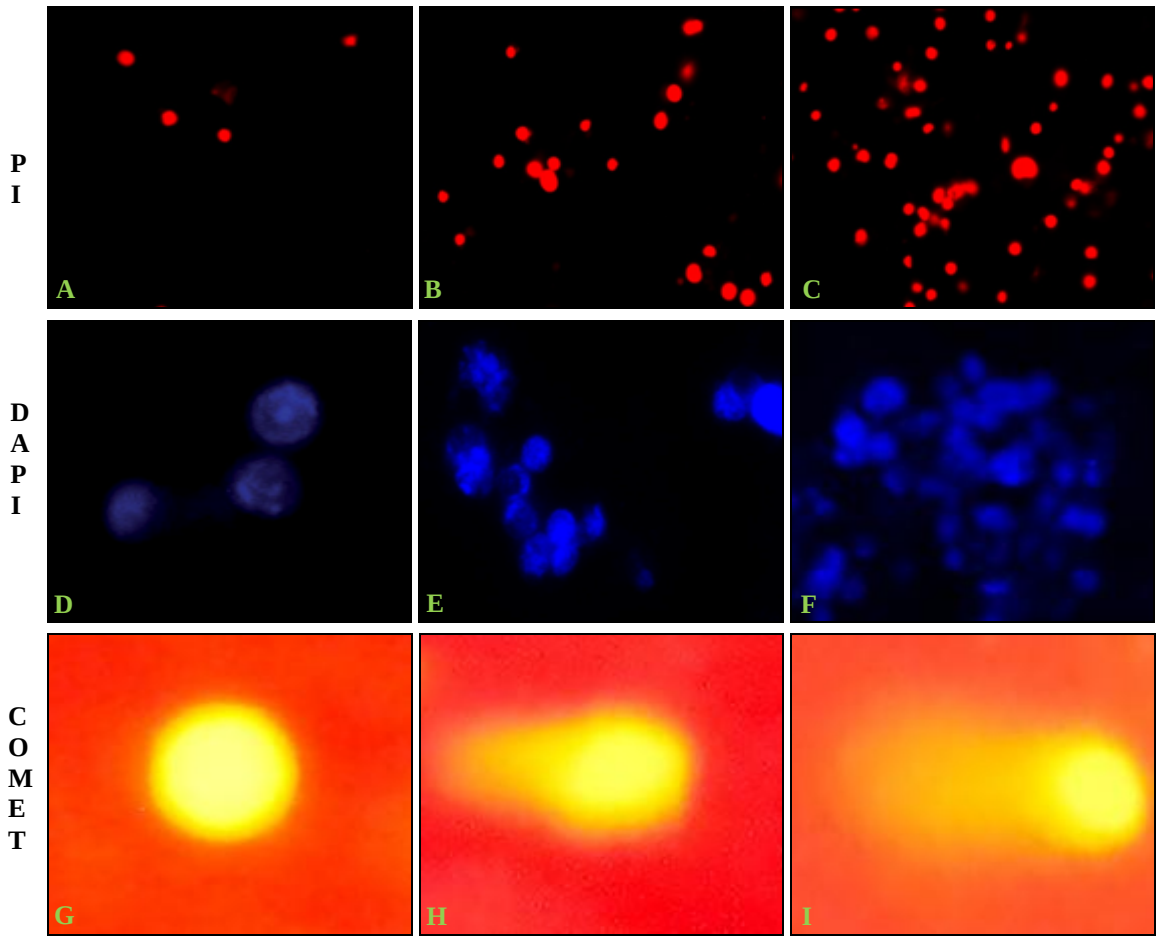

FIG. 7: CELLULAR DEGRADATION IMAGING OF PURIFIED BIOACTIVE PROTEIN TREATED HT 29 CELLS AFTER 24 h INCUBATION USING PROPIDIUM IODIDE, DAPI STAINING (20X MAGNIFICATION) AND DNA DAMAGE INDUCING EFFECT OF BIOACTIVE PROTEIN ON HT 29 CELLS (40X MAGNIFICATION) BY COMET ASSAY; (A, D & G) CONTROL, (B, E & H) 20 µg/ml, (C, F & I) 100 µg/ml

Cells treated with IC_{50} concentration showed 21.93 tail DNA (%). However, control had only 0.81 tail DNA (%). The tail movement (TM) and olive tail movement (OTM) determines the extent of oxidative DNA damage of the control and treated cells. The results depicts in addition to tail DNA (%), the IC_{50} concentration has a TM of 12.71 and OTM of 14.69 which are appreciable comet scores for DNA damage **Table 3**.

TABLE 3: COMET ASSAY PARAMETERS OF BIOACTIVE PROTEIN OF CATFISH MUSCLE USING CASP SOFTWARE

Parameters	Control	Bioactive protein (µg/ml)	
		20	100
Comet length (in pixels)	241	149	210
Head Length (in pixels)	225	91	103
Tail length (in pixels)	16	58	107
Head DNA (%)	99.18	78.06	64.77
Tail DNA (%)	0.81	21.93	35.22
Tail movement (TM)	0.131	12.71	37.69
Olive tail movement (OTM)	0.87	14.69	33.04

DISCUSSION: Cancer is considered to be one of the most deadly diseases in the medical field. Apart from the preventive therapies, it's important to find a curative measure which holds no loopholes and acts accurately and precisely to curb cancer [21]. The drug development from food derived sources like proteins, carbohydrates and lipids are relevant as it usually furnish drugs of reduced side effects. Thus this is a pioneer work attempted to isolate and characterize the bioactive protein from *T. dussumieri* muscle and evaluate the anti-proliferative effect on HT 29 cells.

According to Boren *et al*., [22] and Kilkuchi *et al*., [23] the gold fish white muscle protein analyzed by SDS-PAGE also resolved protein bands ranging between 6.5 to 205 kDa. The present finding was in line with the previous report of 24 for the fractionation of bovine muscle proteins by DEAE cellulose column chromatography. Matrix Assisted Laser Desorption/Ionization–Mass Spectrometry (MALDI-MS), introduced by Hillenkamp and

Karas 25 is now widely used in proteomics studies. The isolated protein contains Tumor necrosis factor 3 alpha (TNF-3a) peptide which could be responsible for the anticancer activity of the isolated protein. Very recently, a third isoform of TNF is reported, which has low homology to the previously cloned carp TNF's and was named as TNF-3a and its structure has been described [26].

Major histocompatibility complex (MHC) molecules were cell surface-expressed, highly polymorphic, heterodimeric glycoproteins, which initiate specific immune responses through binding peptides produced by degradation of other proteins [27]. In teleost fish species, the class I and II loci form separate linkage groups. Moreover, the class II loci are organised in at least two different groups [28]. According to Ottova *et al*., [29] the class II MHC molecules and its allelism can predict the genetic lineage and phylogenetic relationships of species. The isolated 13.285 kDa protein showed 4 identical peptides of MHC class II beta chain of *Rutilus rutilus* (common roaches). The putative peptide homology with three different proteins in freshwater *Tetraodon nigroviridis* (puffer fish), throw light upon the evolutionary link between the freshwater and marine fishes. Thus, the literature supports that the isolated protein may have its origin from acanthopterigii class.

In drug delivery, viability assays are vital steps as it explains the cellular response to the drug. Also, they give information on cell death, survival and metabolic activities. MTT assay was used to assess the effect of isolated bioactive protein on proliferation of HT 29 cells. Similar result of inhibitory effect was observed by Oberdor [30]. In the present study, the cellular degradation was clearly observed in HT 29 cells treated with purified bioactive protein after stained with PI and DAPI. TNF-3a factors have potential bioactive motifs as drug carriers for molecules with a low bioavailability or stability. In this regard, the membrane impermeable PI assisted as a model substance. The possible explanation is that free PI could not overcome intact cell membranes because of its chemical composition. In contrast, its binding to the bioactive protein allows a time-dependent entry into the cells. Nevertheless, the TNF-a doses required to cure tumor-bearing mice lead to normal tissues injury and eventually may cause lethal shock syndrome. This toxicity implies severe limitations for the therapeutic use of TNF-a [31]. But its effect in the microgram level can provide a successful administration in the molecular and subcellular mechanisms. Similar results were observed by Arulvasu *et al*., [10] in A549 cells treated with both crude and partially purified protein from mucus of catfish, *T. dussumieri*.

Aptamers are a special class of nucleic acids that can specifically bind, with high affinity, to a wide array of target molecule [32]. DAPI forms a fluorescent complex preferentially by attaching to the minor groove of AT-rich DNA sequences. Thus, DAPI has been used as a specific aptamer for DNA labelling. DNA curvature has a main role in the recognition of both regulatory and structural proteins, as demonstrated by a number of studies on the molecular aspects of fundamental biological processes such as transcription and chromatin assembly [33]. Thus, the presence of charged ions and amino acids in the isolated bioactive compound may go and bind to DNA thus influencing the DNA curvatures and stability.

Apoptosis is a major form of cell death, initially characterized by the series of stereotypic morphological changes [34]. The most common change is the DNA fragmentation, which is relatively a late event during apoptosis. Cell death detection using comet assay allowed the detection of DNA comet tails for the detection of extent of DNA damage using a DNA-specific fluorochrome PI, which can detect apoptotic cells [35]. An important aspect of DNA damage is the formation free radicals which mainly influence the single strand break and olive tail movement observed in the present study revealed that the generated Reactive oxygen species (ROS) favours the DNA damage leading to cell death. Reports highlight that reactive hydroxyl radicals released by the drugs attack cellular components such as DNA, proteins and lipid to cause various kinds of oxidative damages. DNA damage was not observed in the control cells, as the halo surrounding cell nuclei was clearly visible. ROS are involved in TNF-a induced cell killing [31]. TNF- a is a multifunctional cytokine which were involved in apoptosis and cell survival. TNF-a activates Tumour necrosis factor receptor 1 (TNFR-1) family of proteins, especially NF- kB, which could regulate cell proliferation and

apoptotic death of cancer cells. Moreover, TNF-a improved the accumulation of chemotherapeutic drugs [36]. Thus, the comet assay results of 13.285 kDa TNF-3a like protein confirms similar properties as previously reported.

CONCLUSION: In conclusion, thus, the present investigation reveals that the edible marine catfish *Tachysurus dussumieri's* 13.285 kDa muscle protein possess a potential *in-vitro* anti-proliferative activity against HT 29 human colon adenocarcinoma cell line. This might be due to presence of sequences homologous to TNF-3a factor which gave distinct nature and characteristics to the isolated protein.

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