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EXTRACTION AND UTILIZATION OF FISH SCALE-DERIVED GELATIN IN 3D PRINTING: A SUSTAINABLE APPROACH

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Keywords:

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ABSTRACT: Freshwater fish scales are an abundant and versatile source of high-quality gelatin, with benefits including improved thermal stability, reduced risk of endotoxic shock and zoonotic disease transmission. This work outlines characteristics of gelatin extracted from fish scale and its applicability in 3D bioprinting. The extracted gelatin has molecular weight ranging from 250-280 kDa and is >99% pure, with total impurity content <20ppm. A high bloom strength of 250g was obtained, demonstrating higher gelling ability under external stimuli. Proximate study shows encouraging biological characteristics; it is non-cytotoxic and devoid of any possible microorganisms, suggesting that it is suitable for biological applications. The gelatin also demonstrated cell viability of 146%, suggesting a favourable effect on cell growth. *In-vivo* studies showed no skin irritation or abnormal reactions after 72h of observation, confirming its potential use for biocompatible scaffold. Further, the study focused on optimizing synthesis of cross-linked gelatin to create an ink suitable for 3D bioprinting. Critical analysis was done on the optimized concentration range (5-20%) for the creation of ink as well as the uniaxial compression properties, swelling behavior, and biodegradations.

INTRODUCTION: Gelatin is a natural, translucent, colorless, and flavorless biopolymer derived from the partial hydrolysis of collagen. This collagen is extracted from animal bones, scales, hides, skins, and connective tissues.

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Gelatin is water-soluble and can either form gels or remain non-gelling. It is also sensitive to temperature, making it versatile for various applications [1].

It is composed of amino acids joined together by amide linkages in a long molecular chain, which perform a crucial function in the building of connective tissue in humans. It is widely used in the food and pharmaceutical industry due to its physical and functional properties [2]. Majority of the commercially available gelatin is derived from mammalian source (mainly porcine or bovine).

It is reported that more than 70% of the collagen and gelatin available worldwide is from porcine, followed by bovine [3]. However, the consumption of gelatin from mammalian sources contradicts with many ethnocultural, religious beliefs and is also associated with the risk of infection by prions like mad cow diseases and bovine spongiform encephalopathy (BSE). In this regard, people are skeptical about its usage and therefore an increasing demand and need for alternative sources.

Fish gelatin on the other hand has the potential to be a valuable alternative to mammalian gelatin due to its potential health benefits and unique properties [4]. Compared to mammalian gelatin, fish gelatin also has a lower risk of zoonotic diseases and no special dietary requirement. Also, as the edible part of fish is mostly meat, fish processing industries produce large quantities of inedible parts such as skin, bones, viscera, and scales and due to underutilization of these byproducts they are dumped in the environment, adding to the environmental pollution. Considering the Indian scenario, the fish wastes have exceeded 4 million metric tons in 2018 and this figure is increasing every year [5]. Utilizing fish scales as a source for gelatin production presents a promising opportunity for value addition to this by-product [6].

By utilizing waste-to-wealth technology, we have successfully scaled up gelatin production from freshwater fish scales. Fish scale gelatin offers several advantages over other sources, including a high content of imino acids essential for collagen synthesis, low fat content, and high gel strength. Its versatility allows for applications in various industries such as healthcare products, beverages, nutrition bars, tablets, meat products, and gummies. Moreover, the use of fish scale gelatin promotes sustainability by repurposing fishing industry by-products, reducing waste, and fostering environmentally friendly practices.

Many studies have been devoted to the extraction of gelatin from cold-water fishes like salmon and cod, but this gelatin has been reported to possess inferior functional qualities than mammalian gelatin. However, some recent studies have indicated that gelatin extracted from skin and scales of tropical and sub-tropical warm water fishes like tuna, catfish, perch exhibit similar properties to mammalian gelatin. In the present work, we will discuss about utilizing scales of Indian major freshwater carps Rohu (*Labeo rohita*) and Catla (*Labeo catla*) for the extraction of good quality and quantity of gelatin. 3D bioprinting has become an important part of tissue engineering for customized patient specific applications in complex structures like hearts and livers that could be used to save lives and improve the quality of life for patients suffering from serious diseases and injuries [7].

Gelatin plays a crucial role in 3D bioprinting due to its unique properties, particularly in the form of gelatin methacrylate (GelMA). GelMA is probably the most used and widely studied modified gelatin for bioprinting. Before printing any constructs, an optimal bioink must be selected. Bioink is a combination of biocompatible biomaterial with specific type(s) of cells depending upon the envisioned tissue that one needs to be augmented or replaced. There is no universal bioink and therefore creation of an ideal bioink is a first crucial step that needs to be optimized for different target cells/tissues. The biomaterial used in the creation of ink should be biocompatible with ideal physio-mechanical and biological characteristics. It should also be porous enough to allow the embedded cell to exchange nutrients and oxygen with their environment. For some of these reasons, hydrogels are particularly the biomaterial of choice for 3D bioprinting application [8].

Among various hydrogels, gelatin is a natural hydrogel and most importantly its chemical similarity to the extracellular matrix facilitates the easy recruitment of cells and their reorganization into living structures. Further, fish gelatin is preferred for 3D bioprinting over other types of gelatin due to its properties like nontoxic, biodegradable, biocompatible, has low immunogenicity, immune to zoonic and religious problems.

Fish gelatin being a cold-adapted gelatin has technical advantages over warm-adapted gelatin for biofabrication applications. Fish gelatin has superior thermal stability and is easy to dissolve and handle. Some of these properties makes it a reliable alternative to gelatin from other sources [9]. Thorough chemical, microbial validation of extracted gelatin intended for human consumption

and other quality requirements to qualify as a medical material was done in accordance with the monograph of European Pharmacopoeia (GME) ad ISO standards [10]. Further, the derived fish scale gelatin has been comprehensively investigated for its biocompatibility and potential use in tissue engineering applications, indicating its suitability as a biomaterial for 3D bioprinting.

EXPERIMENTATION:

Materials: Gelatin- type A was extracted from freshwater fish scales by acid hydrolysis using a pilot scale facility as detailed out in **Fig. 1.** Raw materials like Sodium Chloride and Sodium Hydroxide were purchased from Merck for the extraction of Gelatin. While ink for 3D bioprinting was made utilizing Methacrylic anhydride (MA), and 2 – hydroxyl - 4ť - (2-hydroxyethoxy) – 2 - methylpropio-phenone (Irgacure2959) purchased from Sigma-Aldrich. Collagenase type II (>125 U/mg solid) was purchased from MP Biomedicals, India. All chemicals were of analytical grade and were used without any further processing.

Preparation of Gelatin: As gelatin is a denatured form of Collagen, hydrolysis of Collagenous (one word) raw material was performed as the first important step for gelatin production. The collagen hydrolysis is done either by an acid or alkali. As acid hydrolysis is considered better and sustainable due to faster processing time, better yield with good bloom strength and low environmental impact, we have used the acid way of hydrolysis where the pH is maintained between 6-6.5 and the acid hydrolysed gelatin is known as Type A gelatin. Depending on the degree of hydrolysis, gelatin can be produced in both Gelling (Type G) and Non-gelling type (Type F) forms. The extraction of gelatin was done from dried freshwater fish scales.

It is followed by the removal of slime and dirt wherein the scales are washed, softened, and processed using Sodium Hydroxide and then again washed thoroughly with DI water. The washed, softened, and dried scales are then subjected to chemical pre-treatment with dilute acetic acid at 80°C.

This is done to destabilize triple-helix through a disruption of hydrogen bonds and some covalent cross-links, thus enhancing protein solubilization. Gelatin hydrolysates are then filtered through a nylon filter of 0.3-1mm pore size **Fig. 1**. The extracted liquid is immediately stored at -80 °C for 12 h followed by lyophilisation. Then the filtered solution is dried and crushed according to the requirement. The reaction temperature, pH and the extraction process are exclusively designed and optimized to achieve the maximum yield with low batch to batch variation. The dried gelatin also known as FS (fish scale) gelatin is then used for further characterization.

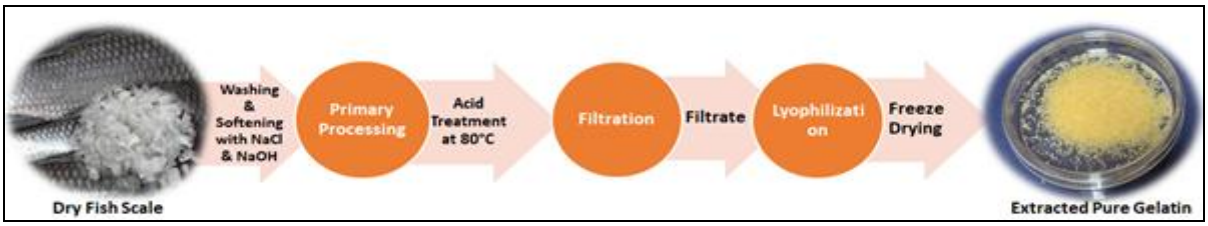

FIG. 1: PROCESS FLOW CHART: EXTRACTION OF GELATIN FROM FRESHWATER FISH SCALE

Preparation of GelMA: The crosslinked gelatin (GelMA) preparation and testing of hydrogels were done according to previously reported optimized process as schematically represented in **Fig. 2** [11].

Briefly, 10% gelatin from fish scale (150-250g bloom strength) was dissolved in phosphate buffered saline (PBS) in a beaker with constant stirring at 50 °C for 20-60 min to promote gelatin dissolution. After being cooled to50 °C, 8% (v/v) Methacrylic anhydride (MA) was added dropwise into the gelatin solution over a period of 20 min and allowed to react for another 3 h. The reaction media was diluted four times using PBS to stop the reaction.

The solution was dialyzed using a dialysis membrane (Sigma-Aldrich; MWCO 12400) for 4 days at 37°Cto remove salts and unreacted MA and then lyophilizedat -80 °C to get white GelMA foam that was stored at -80°C until further use.

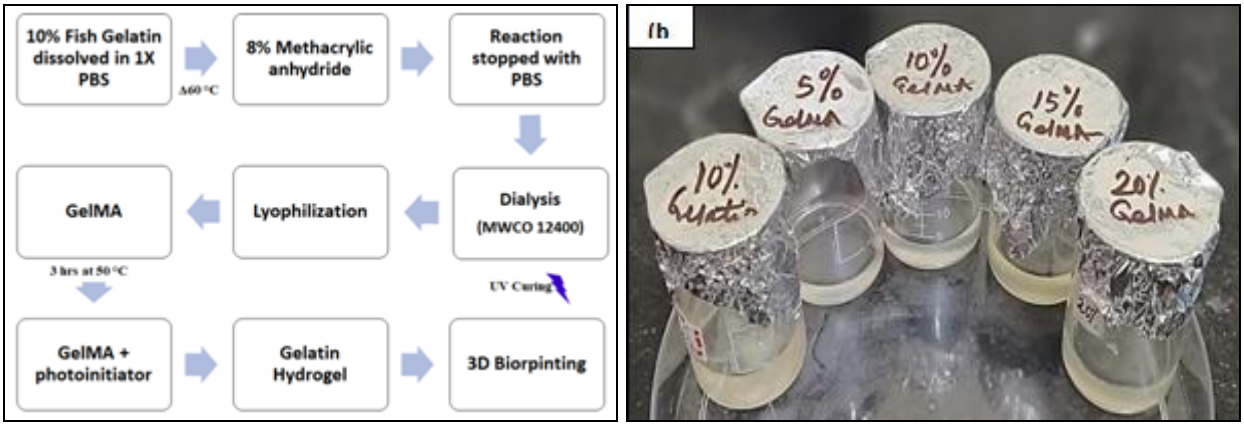

FIG. 2: (A) FLOW CHART SHOWING THE PROCESS INVOLVED IN GELMA PREPARATION, (B) DIFFERENT CONCENTRATIONS THE GELMA FORMULATIONS

Methods:

Physical Characterization of FS Gelatin: All physical analysis like colour, taste, odour was performed by organoleptic evaluation. The turbidity of 20% gelatin solution was measured using a nephelometer and it was expressed in FTU (Formazin Turbidity Unit). The elemental analysis was done by ICP MS- 7700 Agilent Technology. A rapid microwave hydrolysis procedure was used for the amino acid determination in FS gelatin. The amino acids were then eluted using C18 column with a flow rate of 0.8 ml/min in HPLC-1200, Agilent Technology. The bloom value of 6.67% gelatin (according to the GME standard) was calculated using Brookfield's CTX texture analyzer in compression mode with distance of 4 mm, speed of 0.5 mm/s, strain at 10%.

Chemical Characterization of FS Gelatin: Moisture, ash content and viscosity of FS gelatin were evaluated according to the Gelatin Manufacturers of Europe (GME) standard. Briefly, 5g FS gelatin was weighed (m_0) and placed in the drying oven at 105 ± 2 ºC overnight. The sample was then transferred to the evaporating dish (m_1) and was left to dry for 18 h. Its weight was measured (m_2) after the sample was cooled to room temperature.

The percentage moisture content was calculated by the below equation:

$$(m_1 - m_2)/m_0 \times 100$$

Similarly, for ash content, 5g of the extracted gelatin was incinerated in a crucible in a muffle furnace at 550 ºC for 20 h. The total ash % was calculated by:

$$m_1/m_0 \times 100$$

Where: m_0 is quantity weighed in and m1 is the quantity weighed out.

Viscosity of 6.67% FS gelatin was dissolved at 65 °C the sample was then poured into the pipette and the viscosity was determined at 60 °C by measuring the flow time of 100 ml of the solution through the standard pipette.

Biological Characterizations of FS Gelatin:

***In-vitro* Cytotoxicity test on Mouse Fibroblasts Cell Lines (ISO-10993-5):** The Cytotoxicity of FS gelatine was evaluated by MTT Assay using L-929 cells (NCTC clone 929: CCL 1, American Type Culture Collection [ATCC] and study was conducted according to ISO 10993-5:2009 (E), Biological Evaluation of Medical Devices-Part 5: Tests for *in-vitro* Cytotoxicity [12-14].

FS gelatin, positive (ZDEC Polyurethane Film (RM-A)) & negative (High Density Polyethylene Film (RM-C))controls were taken in complete culture medium at 200mg/ml concentration and kept at 37°C for 24 h for extraction. The extract was further diluted with the cell culture medium to attain concentration of 12.5%, 25%, 50%, and 100%. 100µl of 200mg/ml concentration of the FS gelatin extract and reference item were added aseptically per well and was incubated for 24 h at 37 °C, 5% CO_2 environment for the treatment. On the day of treatment each plate was examined under a phase contrast microscope to ensure that cell growth is relatively even across the well.

***In-vivo* Skin Irritation Test on New Zealand White Rabbits (ISO-10993-23):** The purpose of

this study is to assess the possible irritation potential of FS gelatin extracts following intracutaneous injection in rabbit [15].

200mg/ml concentration of FS gelatin was extracted both in polar (sodium chloride 0.9% (w/v)) and non-polar medium (cotton seed oil). Extracts were agitated during the extraction period for 72h at 37 °C. About 18 h before treatment, each animal (total 3 numbers) was clipped free of fur from the back and both sides of the spinal column to yield a sufficient injection area.

Each animal was injected with 0.2 ml of polar and non-polar extractions of test item intracutaneously at 5 sites each (10 sites) on each side of spinal cord as per ISO 10993-10:2010 (E) as shown in **Fig. 3**. The animal was observed for possible irritation at 24, 48 and 72 h.

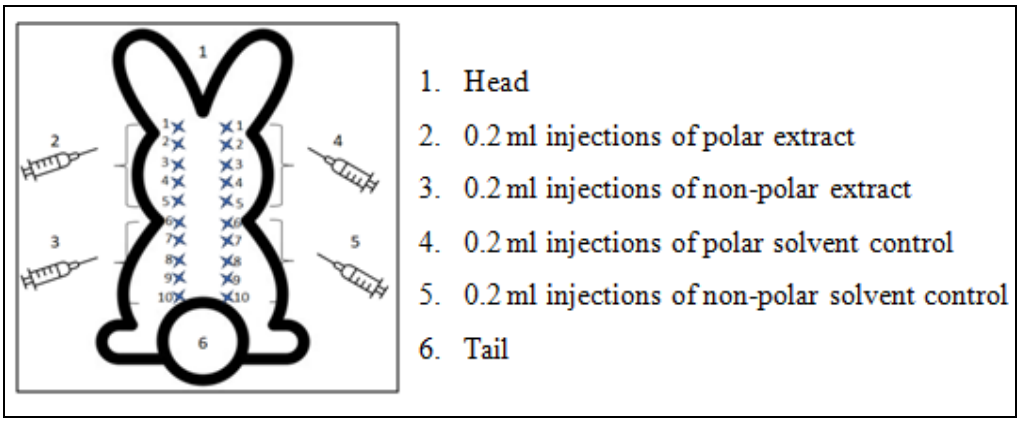

FIG. 3: SCHEMATIC OF INJECTION SITES IN EACH RABBIT

***In-vivo* Skin Sensitization Test on Guinea Pigs (ISO-10993-10):** The objective of skin sensitization study was to assess the allergenic potential of FS gelatin, when applied to the skin of guinea pigs. This study would provide a rational basis for risk assessment of the sensitizing potential of the FS gelatin in humans. The procedure and concentration of sample preparation was same as mentioned above in skin irritation test.

Total of 30 guinea pigs divided into 4 different groups were considered for this test as detailed in **Table 1**. Each group consisted of 5 control animals in polar (G1) and 5 in nonpolar groups (G3) and 10 test animals in polar (G2) and 10 in nonpolar (G4) groups. The animals were subjected to the extracts in 3 different phases *viz.* induction-I (intradermal injection), induction-II (epidermal application) and challenge phase (epidermal application). During intradermal injection the animals were injected with FS gelatin extract and the control animals were injected with the vehicle control [16, 17]. Three rows of intradermal injection (two injections per row) were given to each animal within an approximately 4 x 6 cm boundary of the fur clipped area as illustrated in **Fig. 4.**

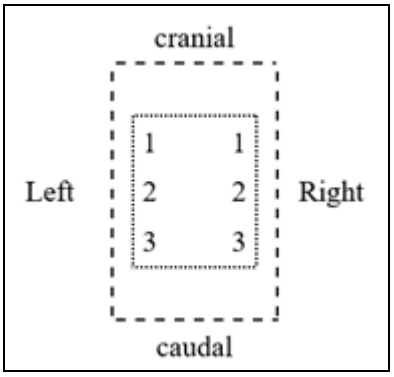

FIG. 4: SCHEMATIC OF SITE OF INJECTION IN GUINEA PIGS

TABLE 1: DETAILED OUTLINE OF PHASES OF SKIN SENSITIZATION INDUCTION

Species/Strain: Guinea Pig/*Albino Dunkin Hartley*			**Number of animals:** 30 **Sex-**Females
		Polar Groups	
Phase	**Administration route/site**	**Day**	**Vehicle: Normal saline**
Induction I	Intradermal/scapular (3 pairs)	0	**Control group (G1-5 females)** FCA: Normal saline(1:1) Vehicle: Normal saline FCA & Normal saline: Normal saline (1:1)

			Test group (G2-10 females): FCA: Normal saline(1:1) Test Item extract FCA & Normal saline: Test Item extract (1:1)
Induction II	Epidermal Application/ scapular	7	Normal saline for control group and 100% concentration of test item extract to test group
First Challenge	Epidermal application / right and left flank	21	Normal saline to right flank and 100% concentration of test item extract to left flank
Non-polar Groups:			
Phase	**Administration route/site**	**Day**	**Vehicle: Cotton seed oil**
Induction I	Intradermal/scapular (3 pairs)	0	**Control group (G3-5 females)** FCA: Cotton seed oil (1:1) Vehicle: Cotton seed oil FCA: Cotton seed oil and Normal saline(1:1) **Test group (G4- 10 females)** FCA: Cotton seed oil (1:1) Test item extract FCA: Cotton seed oil and Test item extract(1:1)
Induction II	Epidermal Application / scapular	7	Cotton seed oil for control group and 100% concentration of test item extract to test group
First Challenge	Epidermal Application / Right and left flank	21	100% concentration of test item extract to left flank and Cotton seed oil to right flank.

Characterization of FS Gelatin Ink and Hydrogel: The master mix for 3D printing ink(homogeneous solutions of different GelMA concentrations with photoinitiator) were pipetted into a 5 mm spacer glass mould and exposed to 365 nm UV light (~17 mW/cm^2 UV; measured using LUTRON UV 340A UV meter) for 90s to allow photocross-linking via free radical polymerization. Immediately after curing, the samples were punched using an 8 mm diameter biopsy puncher. The cylindrical samples were swelled into PBS for 24 h at 37°C to reach the equilibrium swelling state before mechanical testing. The diameter and height of each sample was measured with a vernier callipers. A load–displacement curve was obtained under compression using a micro-UTM analyzer (Mecmesin Multi Test 10-*i*) at a 2 mm/min strain rate using a 500 N load cell. The data were further recalculated for stress and strain values. Similarly, the compressive modulus was determined (n = 4) from the initial slope of the linear region corresponding to 10-20% strain of the stress–strain curve. The data points from the area of interest were plotted in origin, linear curve fitting was done to get the slope value to calculate the modulus.

Degree of substitution (DS) from gelatin and GelMA was calculated by ^{1}HNMR spectra, recorded at a frequency of 400MHz using BrukerAC-400 spectrometer. The functional groups of the synthesized samples were identified by FTIR (Spectrum-One Perkin Elmer) using the KBr-disc method from 400–4000 cm^{-1}. Further the scaffolds were optimized in a 3D printer (Origin$^+$, Avay Bioscience, India).

RESULTS AND DISCUSSION: Proximate analyses of all the physical, chemical, and biological attributes of the extracted FS gelatin and its application as an ink for 3D bioprinting is discussed below:

Physical Properties: The gelatin extracted from fish scale was granular powder with pale yellow appearance as visible in **Fig. 5.** Organoleptic evaluation indicated the FS gelatin to be odorless and tasteless. Deriving a gelatin from fish source without fishy smell is one of the major challenges that was successfully achieved in this work. The turbidity of the sample was found to be 18.6 FTU. The water conductivity of 1% gelatin was found to be 0.17 mS/cm. The bulk density was found to be 0.67 g/ml and the tapped density 0.77g/ml. The extracted gelatin was completely soluble in hot water (60 °C). 25% solution of gelatin in milli Q water had a pH of 7.5 at 25 °C. In summary, thorough physical evaluation of extracted FS gelatin is essential to develop printable, biocompatible and mechanically robust hydrogels for successful 3D bioprinting applications [41]. Though GME standard doesn't specify a target turbidity range for gelatin.

However, it is desirable to have a low turbidity value, indicating a clear and pure gelatin solution. Properties like colour, turbidity, moisture content, density, gelation, solubility etc. can be influenced by factors such as source material, extraction process, residual impurities and microbial contamination, therefore it becomes critical to evaluate these properties if the end application is for any tissue engineering application like bioprinting, regenerative medicine [10].

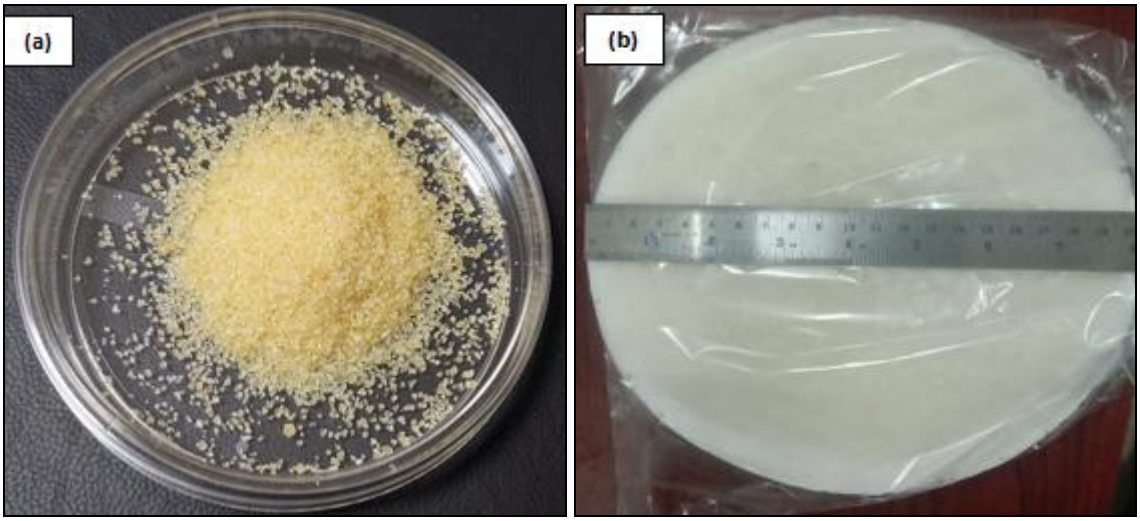

FIG. 5: GRANULAR PALE YELLOW FS GELATIN (A) OVEN DRIED (B) LYOPHILIZED

The extracted FS gelatin had a bloom strength of 250g. Bloom strength is the measurement of the strength or firmness of the gelatin, and its value indirectly reflects the molecular weight of the gelatin. The bloom strength values for Gelatin typically varies in the range of 30-300g. Typically, less than 100g bloom is regarded as low bloom, 100-200g is medium bloom and greater than 200 as high bloom. Higher bloom strength gelatin is considered higher quality because it has longer collagen chains and contains fewer impurities (e.g., nucleic acids, sulphur compounds, non-collagen proteins, and trace metals) [18]. As bloom strength is a measure of gelatin's ability to form gel when exposed to heat or other stimuli, it is crucial property to be considered for 3D bioprinting application. Choosing the right bloom strength is essential for optimizing printability, ensuring desired mechanical properties, and maintaining biocompatibility and functionality.

Chemical Properties: The loss of moisture on drying of FS gelatin was found to be 3.3% and the ash content was found to be 1.0%, which was in accordance with the GME standard. The chemical evaluation and complying to the GME is essential for any biomaterial (extracted gelatin here) for applications related to food, pharmaceuticals, and biomedical fields. Moisture content affects gelatin's gelling ability, viscosity, stability, texture, shelf life, all of which are critical for its performance in 3D printing. Further, high moisture level may lead to microbial growth thereby compromising the safety and quality of the product [19]. Further, ash content is equally important for purity assessment as high ash content indicates presence of some inorganic materials or impurities in gelatin. Similarly, to meet the regulatory compliance for food grade gelatin, ash content should typically be below 2%.

Amino acid screening is an important indicator for the quality of protein or gelatin. Amino acid content and the bloom strength of gelatin is proportional to each other, i.e., gelatin with lower amino acid will have a lower bloom strength and Low viscosity and vice versa [20]. Gelatin being a protein usually contains 18-19 amino acids. The extracted FS gelatin had glycine in maximum with around 27% followed by proline (15%) and hydroxyproline (8%). Glycine helps in arrangement and close packing of the chains while proline restricts its structure confirmation, important for gelation of gelatin. Other amino acids that contribute to gelatin formation were phenylalanine and leucine together comprising around 10%, alanine and arginine as 10 & 8% respectively. Aspartic acid and glutamic acid were found to be 7

and 11%. Since, it lacked tryptophan and was deficient in isoleucine, threonine and methionine, gelatin is not regarded as a nutritionally complete protein. As higher proline and hydroxyproline content are usually responsible for the higher degradation temperature and higher stability of the gelatin therefore the extracted gelatin was found to be more stable [21]. The viscosity of the gelatin is defined as the dynamic viscosity ($\eta = kpt$), calculated as 30 mPa.s.

To qualify the produced gelatin safe to consume, especially for pharmaceutical or food applications, heavy metal screening is of prime importance. These heavy metals are harmful and non-biodegradable substances whose concentration in the body of living organisms is usually higher than the environment due to accumulation at each trophic level of the food chain by the process called biomagnification. Therefore, it becomes crucial to screen extracted FS gelatin for heavy metals especially when the end application is bioprinting because the impurity content will define the biocompatibility, reproducibility, reliability, and patient safety of the product (scaffold). Further, medical application of 3D printed constructs is subject to strict regulatory standards. Ensuring FS gelatin is free from harmful contaminants is essential for meeting these standards and gaining approval for clinical use. All the values for FS gelatin were within the permissible range set by GME rendering this gelatin to be pure and free from any impurities as shown in **Table 2.**

TABLE 2: ICP ANALYSIS EXHIBITING THE PRESENCE OF HEAVY METALS IN FS GELATIN

Heavy Metals	GME Permissible Limit	FS Gelatin (mg/kg)
Arsenic	1	0.014
Lead	5	0.582
Copper	30	4
Zin	30	3.83
Chromium	10	0.3
Mercury	0.15	0.059
Cadmium	0.5	0.037
Iron	30	8.73

SDS page was done to reveal the integrity of the polymer chain of the extracted FS gelatin. The SDS page clearly evidenced clear bands for alpha-I, alpha-II and beta that were compared with standard molecular marker and commercially available bovine gelatin from Sigma. Molecular weight was around 250 kDa as shown in **Fig. 6.**

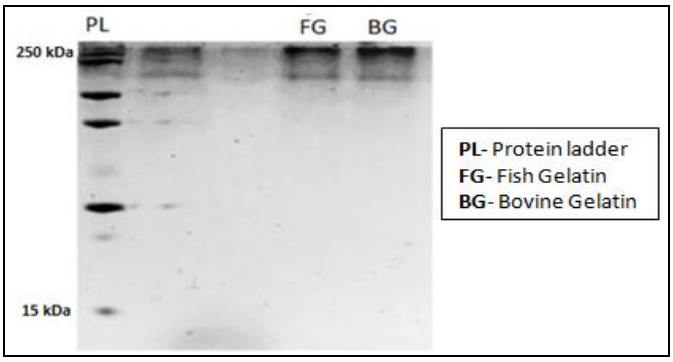

FIG. 6: A CLEAR BAND APPEARS AT 250KDA SHOWING THE PURITY OF THE GELATIN

Screening the microbial load of extracted FS gelatin becomes of prime importance when the targeted application is as a medical material. Testing contamination by some microorganisms like bacteria, yeast and mould is crucial as gelatin being an animal derived product, is susceptible to contamination by various microorganisms during its processing and storage. Bacteria like Bacillus and other related genera possess a serious threat to the Quality of gelatin and its application in 3D bioinks as they can survive extreme pH and temperature conditions during manufacturing by Producing harmful toxins, leading to spoilage. Some of these contaminants are also found to have gelatinase activity that negatively affect the quality of gelatin. Therefore, a guaranteed microbial sterility of the product is of great concern because of its food poisoning potential [22]. Maximum values for edible gelatin prescribed as per Regulation (EC) No 2073/2005, Annex I, Chapter 1, point 1.10 is shown in the **Table 3**. FS gelatin was found to be free of all the potential microbes thereby meeting safety standards and regulatory requirements.

TABLE 3: SHOWING THE TOTAL MICROBIAL LOAD OF TESTGELATIN IN COMPLIANCE WITH THE DIFFERENT STANDARDS

Microbes	Food Regulation EC/2073/2005	European Pharmacopoeia	GME requirements of edible gelatin	FS gelatin
Salmonella	Absent/25g	Absent/10g	Absent/25g	Absent/25g
Total aerobic Microbial count	-	max 1000CFU/g	<1000 CFU/g	160 CFU/g
E. coli	-	Absent/g	Absent/10g	Absent/10g

Anaerobic Count	-		<1000 CFU/g	Absent
Yeast & Molds	-	Max 100 CFU/g	-	CFU/g

Biological Properties of Gelatin:

Cytotoxicity: The comparison of % cell viability test item, negative control and positive control with vehicle control suggested that, in positive control the % viability was minimal. In negative control the % viability was observed as 90.07%, while in test item, the % viability of the highest concentration (100%) was 146.73% **(Fig.7)**. The test concluded FS gelatin, as "non-cytotoxic". It may be noted that the cell viability in all the four concentrations is higher than the negative control suggesting that gelatin is aiding the cell division, growth, and proliferation. Increased cell viability is observed due to gelatin's ability to mimic extracellular matrix, providing a supportive environment for cell growth and differentiation. Better cell adhesion, nutrient availability, biocompatibility, structural support is some of the ways by which gelatin aids cell viability compared to the control.

However, cell viability is better in lower gelatin concentrations than higher as observed in **Fig. 7.** The probable reason for this could be the higher concentration that may lead to protein aggregation or osmotic stress and can restrict the cell growth and proliferation.

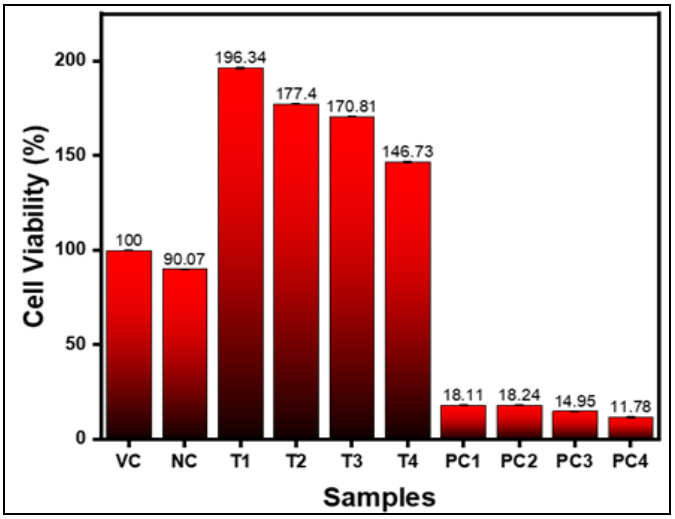

FIG. 7: CELL VIABILITY% ACCESSED THROUGH MTT ASSAY (T=TEST ITEM CONCENTRATION (T1= 12.5% T2-=25%, T3=50% AND T4=100%), PC=POSITIVE CONTROL CONCENTRATION (PC1= 12.5% PC2-=25%, PC3=50% AND PC4=100%), VC= VEHICLE CONTROL (CULTURE MEDIUM WITH CELLS) AND NC= NEGATIVE CONTROL)

Skin Irritation Test: No abnormality or mortality was detected during the experimentation period. No treatment related signs of toxicity and dermal irritation reactions were observed during the observation period of 72 h of both limit test and confirmatory test.

Body weights of all the three animals were found to be normal during the experimental period. Gross pathology of all the vital organs after 72 h of the limit test and confirmatory test were found to be normal, suggesting no incidence of irritation or side effects of the intracutaneously administered FS gelatin extracts up to 72 h of injection **Fig. 8**.

These tests are of biological relevance as they are designed to mimic human skin responses, making them relevant for assessing the irritancy of medical materials like fish scale gelatin, which may interact differently with skin compared to other substances. Overall, skin irritation testing is a critical component of the biological safety evaluation for medical materials, ensuring that they are safe for consumer use.

Skin Sensitization Test: General observations suggested no clinical signs and mortality during the test period. The body weights of all the animals were normal during the experimental period. Repeated intradermal inductions didn't show any adverse effects in the control and treated animals for 24 days observation.

After epidermal induction also there were no adverse effects in both control and treated animals. Even after the first challenge test, there were no adverse effects in the control and treated group animals at 24 and 48 h of observation after patch removal. Overall, gross necroscopy results also suggested no abnormality or sensitization by the application of the FS gelatin for a long period of 24 days **Fig. 9.**

And the rating of allergenicity according to Magnusson and Kligman test in guinea pigs (females) contact hypersensitivity classified FS gelatin to be a weak sensitizer, Grade 0. Grade 0 gelatin typically shows minimal or no irritation,

making it suitable for sensitive skin applications. Formulations or bioinks made of such type of non-irritant gelatin often results in fewer adverse reaction compared to others [24].

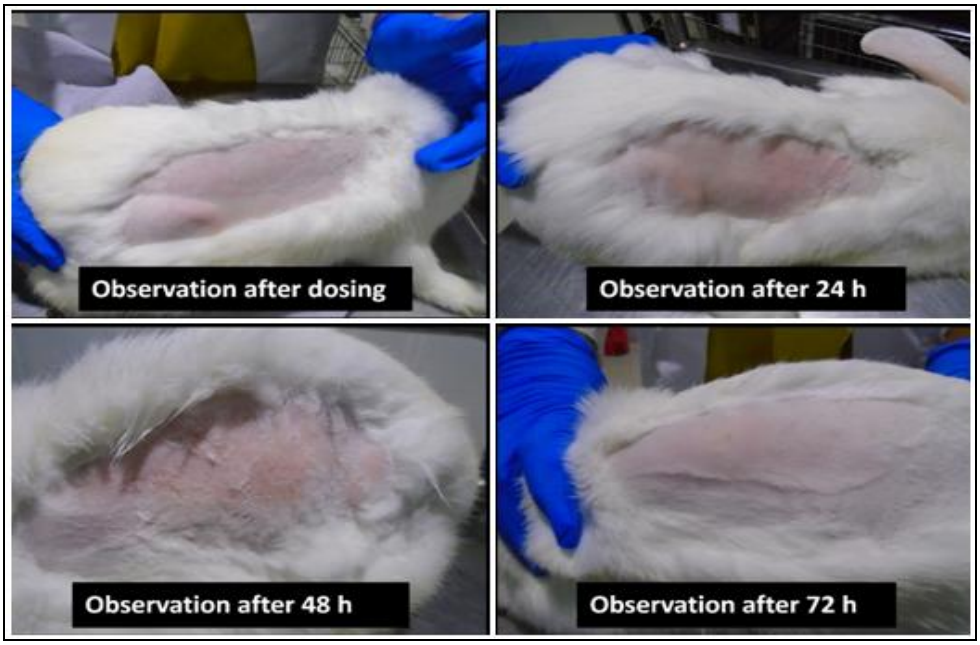

FIG. 8: RABBIT SHOWING NO SIGN OF IRRITATION OR SIDE EFFECTS UP TO 72 H OF INSPECTION

Validation and Application of Fish gelatin as an ingredient of ink for 3D Bioprinting: It is the tunable and versatile physicochemical properties and thermosensitive nature of gelatin that allows us to explore its application in an emerging and trend setting technology like 3D bioprinting. Gelatin to GelMA formation or crosslinking was confirmed by ^{1}HNMR and FTIR. The intrinsic strength of gelatin was checked by mechanical testing. Further, to ensure the effectiveness of scaffolds for biomedical application as well for optimizing their design and performance its swelling behavior and biodegradation was checked. **Fig. 9** shows the degree of methacrylic substitution (DS) in the gelatin polymer chain. The DS can be manipulated by playing with temperature, time, type of solvent and concentration ratio of gelatin to crosslinker.

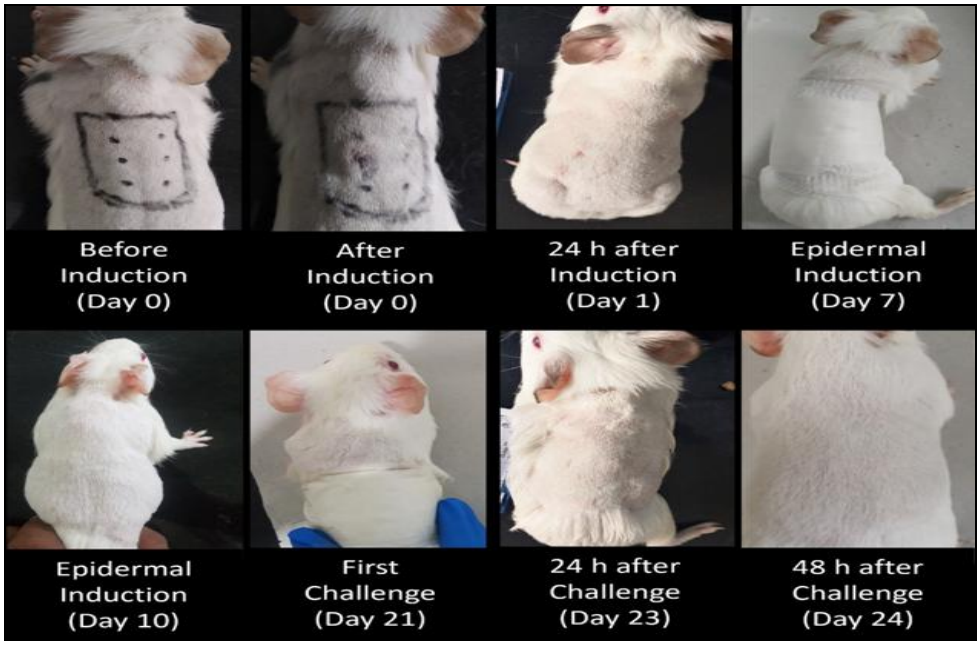

FIG. 9: GUINEA PIGS SHOWING NO HYPERSENSITIVITY TOWARDS FS GELATIN UP TO 24 DAYS OF INSPECTION

The DS was estimated using NMR spectroscopy recorded at a frequency of 400 MHz. Considering the GelMA spectra, the protons of the methacrylic group (H_z) appeared at 5.4 ppm (H_b) and 5.6 ppm (H_a), suggesting the methacrylic substitution in gelatin. Moreover, the intense signal appeared are δ = 1.8 ppm, assigned the methyl protons (H_y) of the substituted methacrylic group. The intensity of Ɛ-NH_2 proton signal of lysine (H_x) significantly decreases due to methacrylic derivatization [12]. The DS of methacrylamide-modified gelatin can be quantitatively analyzed using the peak area ratio of modified amino groups (lysine methylene) to the main amino group peak at 2.9 ppm. The degree of substitution (DS) of gelatin and crosslinked gelatin was calculated to be 89 ±3.4% by the below equation, confirming the formation of GelMA with a high degree of substitution. The high DS value also indicates large number of crosslinking sites exhibited by GelMA, critical for achieving a stable hydrogel. Further, the aromatic amino acid peaks play a key role in determining the inherent structure of gelatin, which primarily originates from the amino acid sequence of collagen. These peaks largely remain intact following methacrylation, which is crucial for preserving gelatin's natural biological signals. This is essential for ensuring its biocompatibility and promoting effective cell interactions. Overall, NMR suggests FS Gelatin can be an efficient and sustainable source for GelMA synthesis for 3D bioprinting application [25].

DS% = (1- Peak of area GelMA / Peak of area Gelatin × 100

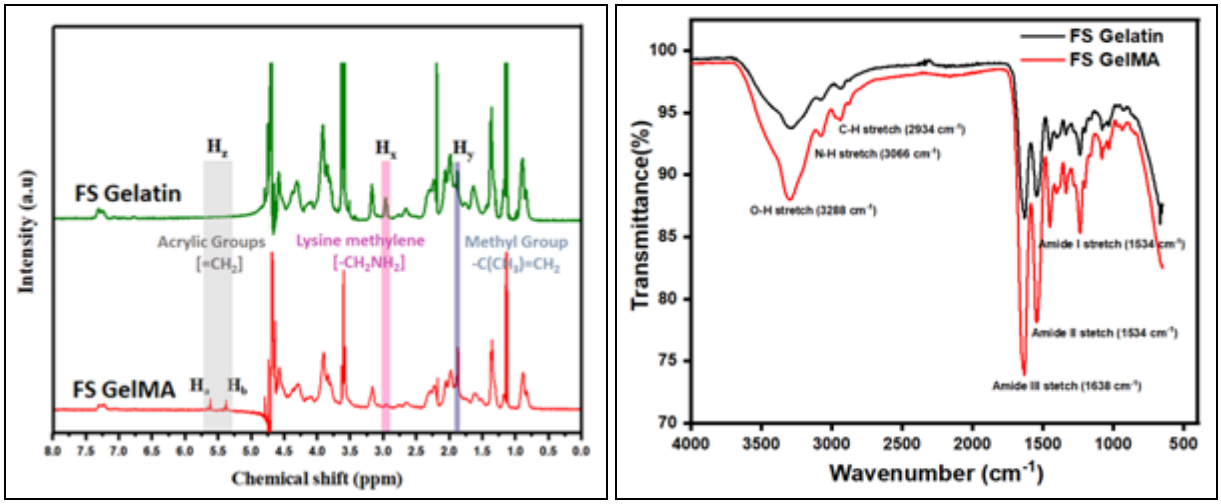

FIG. 10: NUCLEAR MAGNETIC RESONANCE (1H NMR), 10B-FT-IR SPECTRA SHOWING FISH GELATIN AND CROSSLINKED FISH GELATIN (GELMA)

FTIR spectra showed the presence of O–H, N–H and C-H functional groups located at 3283, 3066 and 2934 cm^{-1}, both in gelatin and synthesized GelMA. GelMA had a strong peak of C=O at 1638 cm^{-1}. The double carbonyl stretching peaks for methacrylic anhydride fall in 1751 and 1782 cm^{-1}, these peaks disappeared in GelMA due to the formation of amide bonds [26]. The presence of the main functional groups of the gelatin in synthesized GelMA with shifts in some peak position can confirm the rearrangement in the chemical structure and the successful formation of GelMA (**Fig. 10).**

The compressive stress-strain response shows the viscoelastic behavior of the hydrogel. The elastic modulus of the hydrogel was calculated from the linear elastic region up to 20% strain. This is then followed by a nonlinear part and finally the densification region of the hydrogel where the rapid increment of stress accumulates just before the failure. **Fig. 11** demonstrates the mechanical properties of the hydrogels. The 20% GelMA hydrogel show a typical elastomeric elongation with a maximum stress of 1799kPa, modulus of 296 kPa and maximum elongation up to 74%.

The hydrogel samples (n=4) were immersed in PBS buffer for 24 h; then tested with 500N load cell, at a constant speed of 0.5 mm/min. The compressive modulus and the strength of GelMA increased with increase in the concentration. The tuneable mechanical property of FS-GelMA makes it favourable for 3D bioprinting application with a high printing accuracy, and adequate compressive

strength which in turn enhances cell viability and proliferation. Further, utilizing fish gelatin reduces the risk of disease transmission that is usually associated with mammalian products, particularly in clinical settings where safety is of paramount importance [27].

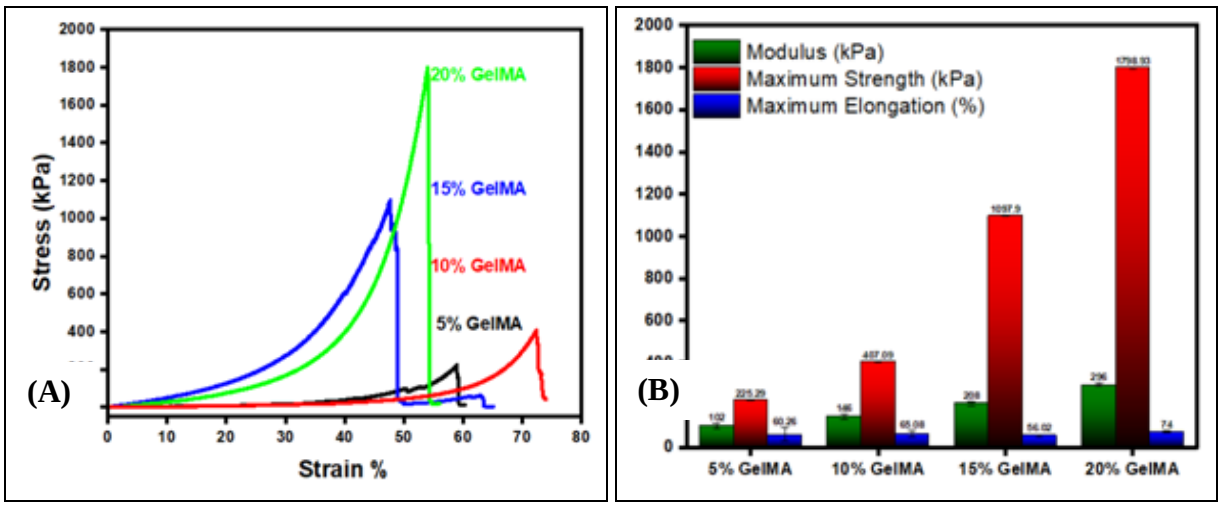

FIG. 11: UNIAXIAL COMPRESSION BEHAVIOUR OF CROSSLINKED HYDROGELS WITH DIFFERENT CONCENTRATIONS OF GELMA (A) STRESS–STRAIN RESPONSE AND (B) COMPRESSIVE MODULUS

The hydrogels stability in terms of its swelling and degradation behaviour is an important factor that needs to be considered for any tissue engineering and medical applications. Porosity of hydrogel-based scaffolds is critical for cell migration, flow of nutrients, growth factors and removal of cellular metabolites during tissue remodelling. Since the water uptake can be directly correlated with the rate of diffusion of water through the porous hydrogel network, the rate of water uptake was determined by rehydrating the lyophilized samples. The rate of water intake and the swelling % can directly be correlated to the porosity and pore volume of the GelMA matrix.The percentage of water uptake of different concentrations of crosslinked GelMA scaffolds was checked at different time intervals of incubation in PBS at 37 °C.

Lower GelMA concentrations exhibited higher percentage of water uptake compared to higher concentration. The respective rate of water uptake by 5, 10, 15, 20% scaffolds were 609, 548, 488 and 433% **Fig. 12**. For better understanding on the kinetics of water uptake, graphs were interpolated using different models and exponential association equations. The graph followed the exponential association model with a high correlation coefficient. This type of model is usually seen in many chemical and biological processes where the outcome is the sum of a fast followed by a slow exponential decay.

$$Y = Y_0 + A_1 (1\text{-}\exp(-K_{fast}*t)) + A_2 (1\text{-}\exp(-K_{slow}*t))$$

Where Y is the water uptake ratio, Y_0 is the water uptake of scaffolds at time 0 (t). K_{fast} and K_{slow} are the two-rate constant, expressed in reciprocal of the X axis time units and A is the fitting parameter.

Similarly, the stability and biodegradation of scaffolds is often desired in tissue engineering and regenerative medicine as it regulates a balance between gradual breakdown of the scaffolds facilitating new tissue integration and regeneration. The enzyme that is mainly responsible for breaking down structural proteins like collagen and gelatin is collagenase and therefore to quantify the rate of degradation of hydrogels, it was incubated in PBS solution containing 2U/ml collagenase II at 37°C. The residual mass of the incubated samples (n = 4) at various time points was measured after lyophilization. The degradation rate of 10% GelMA (complete degradation after 36 h) was more than 15% GelMA (complete degradation after 72 h) **Fig. 12.**

There is a positive correlation observed between swelling and the degradation behavior. Lower the concentration, more is the number of pores and therefore more water uptake. More the number of pores, more accelerated is the diffusion of enzymes or other biodegrading agents into the scaffold matrix, thus faster the degradation rate. Owing to

this, degradation of 5% is more than 10, 15 and 20% GelMA. Different concentrations of GelMA were then formulated into a bioink for 3D bioprinting application. After repeated trials the printing parameters like the print head and print bed temperatures, printing speed, nozzle diameter and the extrusion pressure could be optimized. A predesigned scaffold architecture was used to print the construct using an extrusion-based 3D bioprinter. Temperature control was challenging while printing lower concentrations of bioink because of the thermosensitive behaviour of gelatin. However, uniform extrudability, shape fidelity and structure stability were achieved while printing 15 & 20% GelMA at 22 °C.

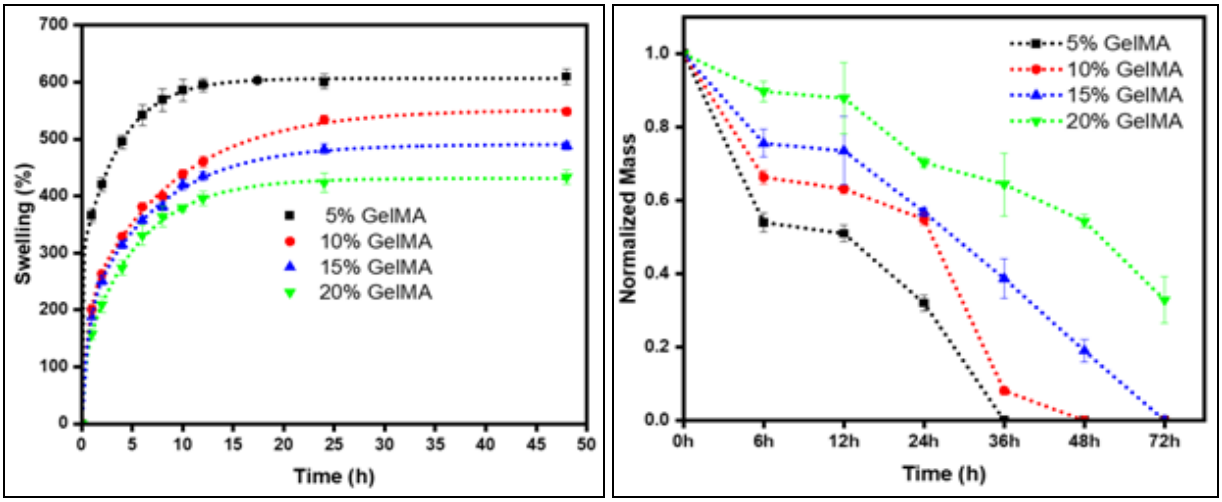

FIG. 12(A) SWELLING STUDY IN PHOSPHATE BUFFER SALINEAT 37°C. (B) ENZYMATIC BIODEGRADATION OFUP TO 72H INCUBATION AT 37°C IN PRESENCE OF COLLAGENASE I-BASEDPHOSPHATE BUFFER SALINE (PBS) SOLUTION OF DIFFERENT GELMA

Fig. 13 shows 20 layered 3D printed construct extruded through a 22G plastic tapered needle with 3 bars pressure and 7% infill density. Single filaments were also deployed with great precision suggesting FS gelatin to be a perfect biopolymer/biomaterial that can be used as an ingredient of bioink. It was observed that higher concentrations printed more rigid structures compared to lower concentrations that could be attributed to the thermal stability of gelatin at higher concentrations. Among all 20% FS GelMA derived from freshwater fish scale gelatin is the suitable concentration for printing 3D constructs.

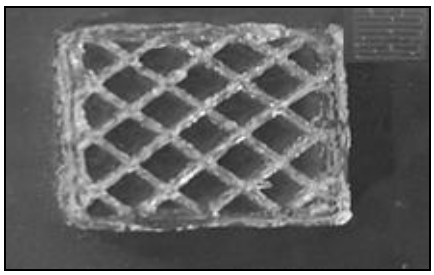

FIG. 13: THE 3D PRINTED 20 LAYERED STRUCTURE USING FS GELATIN

CONCLUSIONS: In the present work, pilot scale gelatin from fish scales were extracted and its 3D printability for the application of scaffold was evaluated. Following conclusions can be drawn from the present work.

- Fish scale gelatin with over 90% purity, 250g bloom strength and with impurity content less than 20 ppm was achieved through a unique approach employed to extract.
- FS gelatin was found to be free of all the potential microbes and non-cytotoxic, indicating its safety for biological use.
- The gelatin demonstrated cell viability of 146%, suggesting a positive effect on cell growth.
- *In-vivo* studies showed no skin sensitization or abnormal reactions up to 24 days of inspection, affirming its potential for biocompatible scaffold application.
- Experimental results indicate that by optimizing the GelMA content, the uniaxial compression properties, swelling behavior, and biodegradation can be tuned to achieve the

required property for tissue engineering applications.

- The FS gelatin-based hydrogel can be precisely fabricated into a predefined 3D scaffold structure by optimizing the printing parameters.

Thus, we conclude that FS gelatin holds great potential for 3D printing applications, as well as for use in other biomedical applications.

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