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# Extraction, HPLC Optimization and *In-Vitro* Anti-Inflammatory Evaluation of Stingray Liver Extract Using RAW 264.7 cell line

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## Abstract

A rapid and sensitive RP-HPLC method was developed for quantitative analysis of hyaluronic acid extracted from the stingray liver and validated using the RAW 264.7 macrophage cells to assess the cytocompatibility of the developed method. The separation was performed by chromatographic method on a Phenomenex Luna C18 column with Britton–Robinson buffer (pH 8.0) as the mobile phase and UV detection at 220 nm. The method showed good linearity (R2 = 0.9981), acceptable recovery (96.6-102.5%), high precision (%RSD < 1%), high robustness, and high sensitivity (LOD and LOQ were 0.001 and 0.003 µg/mL respectively) which met the ICH Q2(R2) guidelines. MTT assay revealed that concentrations up to 100 µg/mL had little effect on cell viability and were found to be less than 10% cytotoxic. The results prove that the method developed is a good approach for routine analysis and also proves that HA produced from the liver of stingrays could be promising for biomedical applications.

**Keywords:** Hyaluronic acid; Stingray liver; Marine biomaterials; RP-HPLC; ICH Q2(R2); Method validation; RAW 264.7 cells; MTT assay; Cytocompatibility.

## 1.INTRODUCTION

Hyaluronic acid (HA) is a linear, unbranched polysaccharide classified as a glycosaminoglycan. While HA is found in various tissues throughout the body, over half of its total proportion is found in the skin. The role of HA in the skin is complex and multifaceted. HA maintains proper hydration, elasticity, and skin firmness, serving as a key extracellular matrix (ECM) component (1) .Unlike other GAG, HA is non-sulfated biomaterial with no capacity to bind a core protein in the formation of proteoglycans.

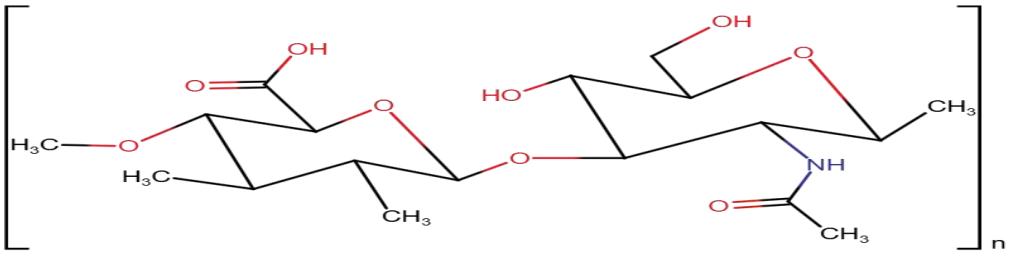



**Fig.1.Structure of Hyaluronic acid**

It was discovered in 1934 and the term “hyaluronic acid” was proposed by Meyer and Palmer, who extracted this biopolymer from the body of a bovine’s eye. They named it hialoide (meaning vitreous) and uronic acid . HA has great biocompatibility and a high affinity for water. These properties make the use of HA in various fields possible . It is commonly used in protective and physiological processes, including in the healing of wounds and burns , in tissue regeneration, for cell differentiation, morphogenesis, angiogenesis, and inflammation(2). HA is involved in the process of a cell’s adaptation to physical and chemical exposure, the process of fertilization, embryogenesis, angiogenesis, inflammation, regeneration and tumor growth(4). HA can be extracted from various animal tissues such as human umbilical cords, rooster combs, bovine vitreous humor, and bovine synovial fluid(5). On the other hand, bacterial fermentation requires highcost infrastructure set up(5). HA derived from animal tissues have naturally high molecular weights and the highest concentration ever reported was from rooster combs which can reach up to 7500 lg hyaluronan/g of that animal tissue (Fraser et al. 1997). Today, although animal-derived HA still remains as an important resource for most HA-based products(6). organisms living in the oceans presents a wealth of potential for developing high-value bioactive substances and biomaterials. Although many biomaterials have been derived from marine organisms in recent decades there is still a great deal of unexplored potential(4). HA has received much attention lately as a natural source of health and beauty, this stab of isolation of HA from marine source was carried out to endow with an alternative source for hyaluronic acid (HA) isolation.(7). Although hyaluronic acid research pursuits ahead in exploring its biomedical perspective, very limited investigations were carried outin their isolation shape view point,furthermore, most ofthe investigations were targeted towards the terrestrial source. To swerve from that, the present study was projected through the marine superstore, where in high molecular weight hyaluronic acid of 13, 65,863 Da was isolated from the liver of stingray Aetobatus narinari(7). Furthermore, these animal tissues can be acquired from animal waste and by-products that would otherwise be discarded, leading to economic and environmental benefit(8). Until now, several different determination methods of HA were developed and most of the reported methods are by

derivatization but the effectiveness of the developed methods was not beneficial for pharmaceutical procedures in terms of being simple, accurate, precise, and sensitive(9). Until now, several different determination methods of HA were developed and most of the reported methods are by derivatization but the effectiveness of the developed methods was not beneficial for pharmaceutical procedures in terms of being simple, accurate, precise, and sensitive.(9). In this study, an attempt was made to find new method cheaper and simple regarding to the limited detection and reproducibility and sensitivity(10). Inflammation is a physiological process that occurs in response to an infectious agent or tissue injury, to restore the homeostasis of the affected tissue(12).However, the process becomes harmful when it is chronic and/ or happens due to autoimmune diseases causing damage to body like cardiovascular diseases, diabetes, neuro-inflammatory diseases, atherosclerosis, rheumatoid arthritis, psoriasis, and graft rejection(13). In vitro models using RAW 264.7 cells are the most commonly applied models when screening for antiinflammatory and immunomodulatory compounds. This is because the RAW 264.7 cell line produces a robust and well-known inflammatory response(12).

## 2. METHODS AND REAGENTS

### 2.1. Chemicals and Reagents

Analytical grade Britton Robinson Buffer , and ultrapure water (Milli-Q, USA) were used throughout the study. Sodium Hydroxide (85%, analytical reagent grade) .Reference standards of Hyaluronic Acid were obtained from USP Reference Standards (Fourtts pharmaceuticals). For the extraction process acetone , sodium acetate buffer (PH 5.5) containing EDTA and L-cysteine , papin for enzyme digestion and ethanol.

### 2.2 Instrumentation

All chromatographic work was conducted on a Shimadzu LC-20AD high-performance liquid chromatography system (Shimadzu Corporation, Kyoto, Japan), comprising an LC-20AD binary solvent delivery pump, a SIL-20AC refrigerated autosampler, a CTO-20A column thermostat, and an SPD-M20A photodiode array (PDA) detector. Chromatographic data were acquired and processed through Shimadzu Lab Solutions software. Analyte separation was achieved on a Phenomenex Luna® C18 reversed-phase column (250 mm × 4.6 mm i.d., 5 µm particle size). The column compartment was thermostatted at 30°C for all runs. Weighing operations were performed on a Mettler Toledo XPE205 semi-microanalytical balance, and pH adjustments were monitored with a Mettler Toledo FG2 pH meter calibrated against NIST-traceable buffer solutions at pH 4.00, 7.00, and 9.21 on each day of use.

### 2.3 Chromatographic conditions

The mobile phase consisted of Britton Robinson buffer 99% and ultrapure water . The pH of the buffer was then adjusted to 8 using Sodium Hydroxide (NAOH) to ensure optimal ionization of the analytes and stable chromatographic performance. The buffer were filtered, degassed to use. The flow rate was set at 0.7 mL/ min, and the injection volume for all analyses was 10 µL. Detection was carried out at a wavelength of 220 nm.

## 2.4 Preparation of standard solution

### 2.4.1 Stock solution

Individual primary stock solutions were prepared by accurately weighing 10 mg of Hyaluronic acid into 10 mL Class-A volumetric flasks. Drug was dissolved in the mobile phase used as the diluent, and the volume was made up to the mark to obtain primary stock solutions containing 1000 µg/mL of Hyaluronic Acid, respectively. A combined working standard stock solution containing 100 µg/mL of Hyaluronic Acid was prepared by transferring accurately calculated aliquots of the respective primary stock solutions into a 10 mL Class-A volumetric flask and diluting to volume with the mobile phase. Calibration standards at the required concentration levels were subsequently prepared by appropriate volumetric dilution of this combined working standard solution.

### 2.4.2 Working Standard Solutions

The nominal analytical concentration (100% test level) was defined as 10 µg/mL for Hyaluronic Acid , corresponding to the target analytical concentrations employed for method development and validation. For linearity evaluation, five calibration levels were prepared at 1, 2, 3, 4, and 5 µg/mL for Hyaluroinc Acid .The preparation of each calibration standard was done by the appropriate volumetric dilution of the working standard solution with the mobile phase.

### 2.4.3 Extraction Procedure of sample

Fresh stingray liver was collected from a local fish market and transported to the laboratory under chilled conditions.

⇩

The liver tissue was washed thoroughly with distilled water to remove blood and adhering impurities, cut into small pieces.

⇩

And treated with acetone to remove lipids and facilitate dehydration. The acetone-treated tissue was then dried completely at oven at 50c for 6hr.

⇩

The dried liver sample was suspended in **100 mM sodium acetate buffer (pH 5.5)** containing **5 mM ethylenediaminetetraacetic acid (EDTA)** and **5 mM L-cysteine**

⇩

Papain was added to the suspension, and the mixture was incubated at **60°C for 24 h** to achieve enzymatic digestion

⇩

the digest was centrifuged at **5,000 rpm for 15 min**, and the resulting supernatant was carefully collected



To precipitate the extracted polysaccharides, **three volumes of absolute ethanol** were added gradually to the supernatant with gentle mixing. The mixture was allowed to stand for **1 h.**



The precipitated material was recovered by centrifugation at 5,000 rpm for 15 min



The resulting pellet was collected, dried to a constant weight, and pulverized into a fine powder

### 2.4.4 Sample Preparation

The sample is collected and dried ,finely powdered and accurately weighed. The 10mg of the powdered sample was accurately measured and transferred into a 10 ml Class-A volumetric flask . And the mobile phase was added and make up to the mark and the mixture was sonicated for 15 min to guarantee the complete extraction of analytes. Once cooled down to room temperature, further 1ml was taken and diluted with 9ml of mobile phase, to make the final concentration of 10 µl .

## 3. Method Validation (ICH Q2(R2))

The validation was carried out in accordance with the ICH Harmonised Guideline Q2(R2): Validation of Analytical Procedures (adopted 2023)(14,15,16)which replaces the previous ICH Q2(R1) and contains clear guidance on reporting measurement uncertainty, range justification and lifecycle considerations. Specificity, Linearity, Accuracy, Precision (Repeatability and Intermediate Precision), LOD, LOQ and Robustness are the validation parameters taken into consideration in this study.

### 3.1. Specificity

Specificity was evaluated by injecting the mobile phase (blank), and the hyaluronic acid reference standard, and the extracted sample were injected separately under the optimized chromatographic conditions.. The chromatograms were examined for the presence of interfering peaks at the retention time of hyaluronic acid. The blank and extraction buffer chromatograms showed no peaks that interfered with the analyte, confirming the specificity of the method. The retention time of the peak obtained from the stingray liver extract corresponded with that of the hyaluronic acid reference standard. Furthermore, peak purity was evaluated using the photodiode array (PDA) detector, confirming that the analyte peak was spectrally homogeneous and free from co-eluting impurities. These findings demonstrated that the developed HPLC method was specific for the identification and analysis of hyaluronic acid in the stingray liver extract under the optimized chromatographic conditions(14,15).

### 3.2.Linearity

Five calibration concentrations were used for evaluation of linearity. For Htaluronic acid the concentrations were 1, 2, 3, 4, and 5 µg/ml. The combined working standard solution was appropriately diluted to obtain each concentration level in five replicate analysis. A calibration curve was drawn for Hyaluronic acid and the linearity was obtained by applying the least square method of linear regression on the peak area – concentration data(14,15).

### 3.3 Accuracy

The standard addition (recovery) method was used to assess the accuracy. Accurately weighed 10mg of Hyaluronic acid extract to be added to three volumetric flasks. Known amounts of reference standards of Hyaluronic acid standards (50%, 100% and 150% of the nominal concentration) were introduced into each flask. The contents were dissolved in a small volume of the mobile phase, sonicated for 20 minutes and diluted to volume with the mobile phase. Solutions were filtered and 20 µL of each solution were injected to HPLC system. The chromatograms were recorded and the % recovery of Hyaluronic acid was computed for each level.

### 3.4 Precision

#### 3.4.1 Repeatability

The %RSD was used for precision: six standard solutions were separately prepared and analysed in a single day by one analyst, with the same instrument and reagent lots, and the upper limit for precision was taken as 2%.

#### 3.4.2 Intermediate precision

Intermediate precision was determined by running the same experiment two times on calendar days where the same experiment was repeated using fresh reagents and standard solutions, and a different analyst conducted the second day analysis. The Combined % RSD (daily and analytical) was calculated to meet the criteria of $\leq 2.0\%$ to pass[16].

### 3.5 Limit of detection(LOD) and Quantification (LOQ)

The signal to noise ratio method (LOD/S/N ratio $\geq 3$, LOQ/S/N ratio $\geq 10$) and the residual standard deviation of the regression line method as recommended in ICH Q2(R2) were used to estimate detection and quantitation limits. In both methods, it was used independently, and cross-checked with the result in each case being taken as the more conservative estimate[15,16,17].

### 3.6 Robustness

The robustness testing involved the ability of the method to withstand moderate, intentional changes to the mobile phase flow rate and mobile phase ratio. The design was a single factor at a time design where each parameter was varied independently at two levels around the nominal value[15,16,17]. The following performance indicators were monitored: retention time, peak area, tailing factor, resolution and calculated assay value.

## 4.Anti-inflammatory assay in RAW264.7 Cells

RAW264.7 cells, which are considered one of the best models for macrophages, an important component of innate immunity and a major cell in the regulation of inflammatory responses (Qie et al., 2022). In the presence of inducers such as lipopolysaccharide, RAW264.7 cells mimic the inflammatory response by releasing or upregulating a variety of inflammatory mediators (Ding et al., 2022). Therefore, RAW264.7 remains the most commonly used in vitro cell model for screening anti-inflammatory actives and studying inflammation.For other cell models, pre-experimentation and appropriate modifications to various experimental manipulations are required. The cytotoxicity of the drug is the first thing that should be considered when assessing the anti- inflammatory ability and MTT and CCK-8 are the methods commonly used by researchers (Gao et al., 2024) (18).RAW264.7cell were obtained from the **National Centre For Cell Science (NCCS), Pune, India.**

### 4.1 MTT assay

MTT is 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is a dye with a yellow color. The enzyme succinate dehydrogenase in the mitochondria of living cells can reduce MTT to blue-violet water-insoluble formazan, which is deposited in the cells (Lin et al., 2012). The amount of formazan produced is proportional to the number of living cells, which is detected by a microplate reader at 560 nm, and then the number of living cells is inferred from the OD value (18).

### 4.2Cytotoxicity study

**Procedure**

- **Cell seeding**
  Cells were seeded in 96-well plates at an appropriate density (typically $5 \times 10^3$ to $1 \times 10^4$ cells per well) and incubated overnight at 37°C in a humidified atmosphere containing 5% $CO_2$ to allow cell attachment.
- **Treatment of cells**
  After incubation, cells were treated with different concentrations of the test compound and incubated for the required time period (usually 24–48 hours).
- **Addition of MTT reagent**
  Following treatment, 10–20 µl of MTT solution (5 mg/ml) was added to each well containing culture medium.
- **Incubation**
  Cells were incubated for 3–4 hours at 37°C to allow metabolically active cells to convert MTT into purple formazan crystals.
- **Solubilization of formazan**
  After incubation, the culture medium containing MTT was carefully removed.
  100–200 µl of DMSO was added to each well to dissolve the formazan crystals.
- **Measurement of absorbance**
  The absorbance was measured using a microplate reader at 570 nm.
  Control wells containing untreated cells were used as the reference for calculating cell viability.

## 5.Results and Discussion

### 5.1 Mobile Phase Optimization

Preliminary method development was carried out to achieve efficient separation of hyaluronic acid with good peak symmetry, reproducibility, and satisfactory chromatographic performance. Different mobile phase compositions and pH conditions were evaluated, and Britton–Robinson buffer adjusted to pH 8.0 with sodium hydroxide was found to provide the best chromatographic response. The optimized conditions consisted of Britton–Robinson buffer and ultrapure water as the mobile phase, delivered at a flow rate of 0.7 mL/min with detection at 220 nm. Under these conditions, hyaluronic acid produced a sharp, symmetrical peak with good baseline stability and reproducible retention time. The optimized chromatogram is shown in **Figure 3**, and these conditions were selected for subsequent method validation.

### 5.2 System suitability

**Table 1**

System suitability parameters and Acceptance criteria

| **Parameters** | **HYALURONIC ACID** | **Acceptance criteria** |
|---|---|---|
| Retention time | 2.4 | |
| Theoretical plates(N) | 3651 | ≥ 2000 |
| Tailing Factor(T) | 1.8 | ≤2.0 |
| HETP | 51.669 | |

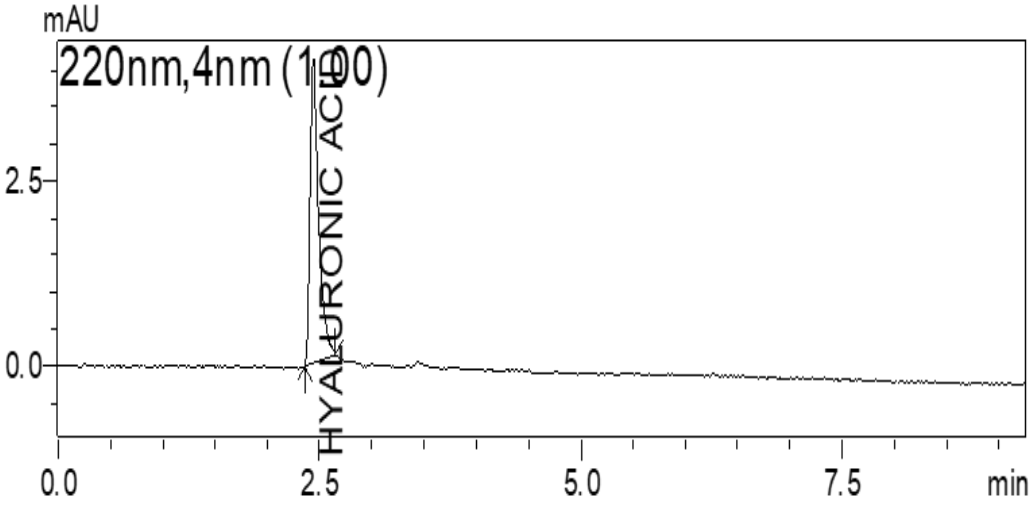
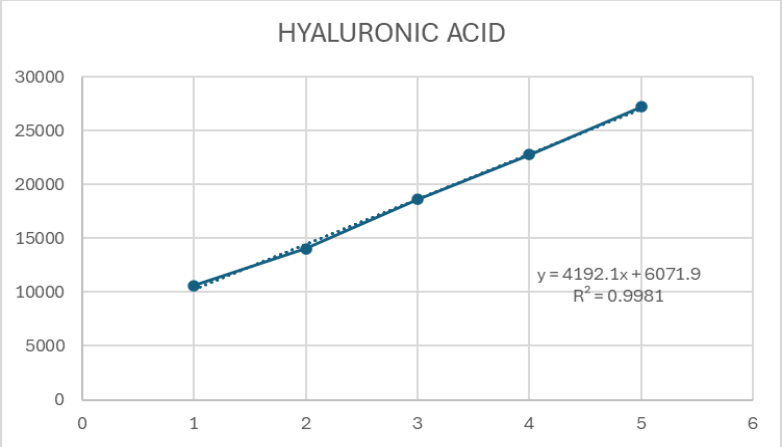


**Fig .2 . Optimized chromatogram for Hyaluronic acid**

### 5.3 Linearity and Calibration curve

The linear range of the proposed method was evaluated by plotting the concentration of Hyaluronic acid in the range of 1-5µg/ml. Excellent linearity was obtained with the correlation coefficient $r^2 = 0.9981$. The regression equations for Hyaluronic acid were y = 4192.1x + 6071.9 respectively which is shown in **Fig .3.**




Concentration (µg/ml)

**Fig .3. Calibration curves of Empagliflozin and Sitagliptin Phosphate**

### 5.4 Accuracy

The developed method was shown to be accurate with the help of recovery studies. The recoveries were found to be satisfactory with mean of 96.6% and 102.5% in the concentration range studied. The results for Recovery studies shown in **Table 2 .**

**Table 2**

Results for Accuracy (Recovery studies) of Hyaluronic acid

| DRUG | SPIKE LEVEL (%) | AREA | AMOUNT OF DRUG ADDED (µg/ml) | AMOUNT OF DRUG FOUND (µg/ml) | PERCENTAGE RECOVERY | %RSD |
|---|---|---|---|---|---|---|
| Hyaluronic acid | 50 | 13147 | 3 | 2.9 | 96.6 | 0.25091 |
| | 100 | 15327 | 4 | 4.1 | 102.5 | 0.2711 |
| | 150 | 28255 | 5 | 4.9 | 98 | 0.17206 |

### 5.5 Precision

The precision of the method was compared by the analysis of the Hyaluronic acid in the concentration range of 5µg/ml, for intra-day and inter-day precision studies. All the %RSD values were obtained to be less than 1.0 % for the analyte under the study conditions which showed good precision and reproducibility of the developed RP-HPLC method.

**Table 3**

Intra-day precision of Hyaluronic acid

| **PARAMETER** | **PEAK AREA OF Hyaluronic acid** |
|---|---|
| Precision | 27197 |
| | 27003 |
| | 27122 |
| | 27356 |
| | 27216 |
| Average* | 27178.8 |
| %RSD | 0.477 |

**Table 4**

Inter-day precision of Hyaluronic acid

| **PARAMETER** | **PEAK AREA OF HYALURONIC ACID** |
|---|---|
| Precision | 27156 |
| | 27182 |
| | 27223 |
| | 27113 |
| | 27206 |
| Average* | 27176 |
| %RSD | 0.15 |

### 5.6 Robustness

The robustness of the developed RP-HPLC method for Hyaluronic acid was checked by introducing small deliberate variations in the mobile phase (Britton Robinson buffer 98 and 100 %v/v), and flow rate (0.6 and 0.8 ml/min). The data for Robustness studies were shown in **Table**

**5**. No significant effect was observed in the optimized analytical conditions on these changes on the chromatography performance, which means that the method is robust and stable in the optimized analytical conditions.

**Table 5**

Robustness studies of Hyaluronic acid.

| **CHROMATOGRAPHIC CONDITION** | | **HYALURONIC ACID** | |
|---|---|---|---|
| | | **AVERAGE PEAK AREA** | **%RSD** |
| **MOBILE PHASE COMPOSITION** | 100% BUFFER | 28210 | 0.35 |
| | 98%BUFFER | 26235.6 | 0.19 |
| **FLOW RATE** | 0.6 | 35823.3 | 0.35 |
| | 0.8 | 22423.6 | 0.27 |

**5.7 LOD and LOQ**

The value for the LOD and LOQ were determined to be

**Table 6**

LOD and LOQ of Hyaluronic acid.

| **PARAMETER** | **HYALURONIC ACID (µg/ml)** |
|---|---|
| LOD | 0.001 |
| LOQ | 0.003 |

The determined LOD and LOQ were low, indicating the good sensitivity of the proposed RP-HPLC method for routine quantitative analysis of Hyaluronic acid.

**6.Anti-inflammatory activity results**

Cell viability was determined based on the absorbance of dissolved formazan crystals produced by metabolically active cells. Higher absorbance values indicate higher cell viability, whereas reduced absorbance reflects increased Cytotoxicity. Treatment with the test compound resulted in concentration-dependent changes in cell viability compared with the untreated control group. The percentage of cell viability was calculated and plotted to determine the cytotoxic effect of the treatment.

**MTT absorption value:**
**TABLE 7**

| **Cells** | **RAW264.7** | | | | **Sample name** | | | **Hyaluronic acid** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Blank** | **0** | **10** | **20** | **30** | **40** | **50** | **60** | **70** | **80** | **90** | **100** |
| **0.043** | **0.945** | **0.871** | **0.87** | **0.866** | **0.864** | **0.861** | **0.858** | **0.849** | **0.846** | **0.843** | **0.841** |
| **0.048** | **0.943** | **0.869** | **0.868** | **0.865** | **0.861** | **0.858** | **0.857** | **0.855** | **0.852** | **0.85** | **0.847** |
| **0.046** | **0.957** | **0.873** | **0.872** | **0.87** | **0.868** | **0.864** | **0.861** | **0.859** | **0.857** | **0.855** | **0.852** |
| **Ave** | **0.948** | **0.871** | **0.870** | **0.867** | **0.864** | **0.861** | **0.859** | **0.854333** | **0.852** | **0.849** | **0.847** |
| **Percentage** | **0** | **8** | **8** | **9** | **9** | **9** | **9** | **10** | **10** | **10** | **11** |
| **Viability** | **100** | **92** | **92** | **91** | **91** | **91** | **91** | **90** | **90** | **90** | **89** |

**IC50 = >100µg/mL**

Cytotoxicity of sample on RAW 264.7 cells determined by the MTT assay. Cells were treated with increasing concentrations of sample (10–100µg/mL) for 24 h, and cell viability was evaluated by measuring absorbance at 570 nm. Data are presented as mean ± SD of three independent experiments (n=3). The $IC_{50}$ value was calculated using nonlinear regression analysis in Graph Pad Prism.

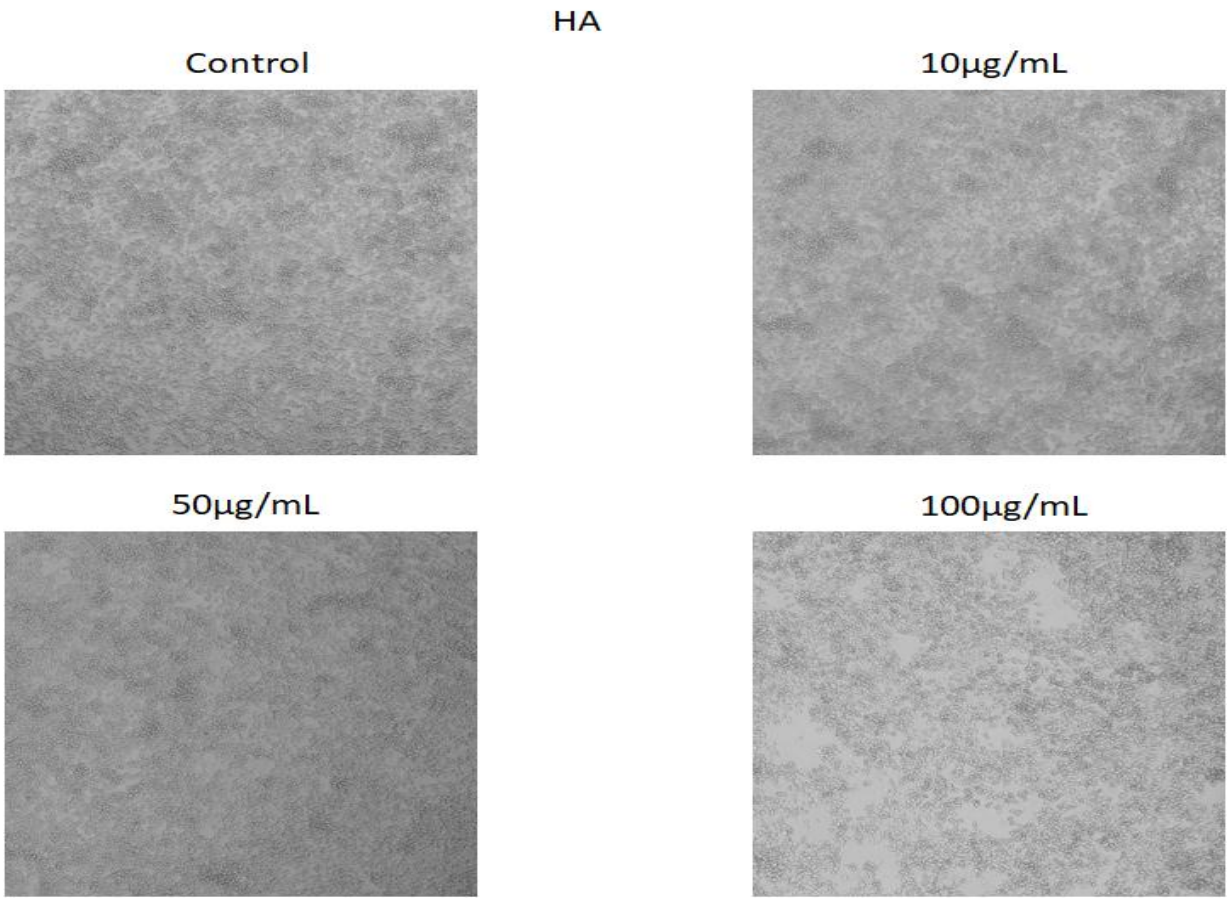



**Fig .4. Scale bar: 100 µm with 10X magnification**

Cytotoxic activity of sample on RAW 264.7 cells determined using the MTT assay. Cells were treated with increasing concentrations of sample (10–100µg/mL) for 24 h. Cell viability was calculated relative to untreated control cells. Data are presented as mean ± SEM (n=3). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs control).

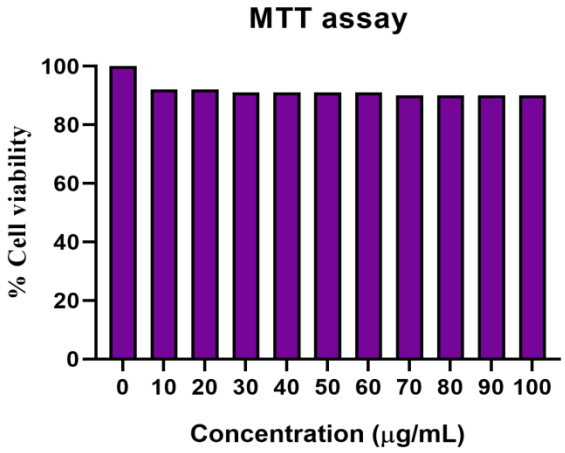



**Fig.5**. Effect of the test sample on RAW 264.7 cell viability determined by the MTT assay

## Conclusion

An RP-HPLC method was developed and validated for the determination of hyaluronic acid extracted from stingray liver based on ICH Q2(R2) guidelines and found to be simple, accurate, precise, sensitive and robust. The optimized conditions of the chromatography guaranteed satisfactory system suitability, excellent linearity ($R^2$ = 0.9981), acceptable accuracy, high precision, low LOD and LOQ, and high robustness, indicating the reliability of the method for routine quality control and quantitative analysis. Additionally, MTT assay results on RAW 264.7 cells demonstrated that the MTT assay value of the stingray liver hyaluronic acid extract was more than 89%, and the $IC_{50}$ was over 100 µg/mL, which implied that the extract is well biocompatible. Based on the findings, it can be concluded that the liver of the stingray is a novel potential source of hyaluronic acid which may be used in biomedical and pharmaceutical applications and is an underutilized marine by-product.

## Declarations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### Credit authorship contribution statement

**Thiruvengadarajan vasanthi srinivasan:** Writing – reviewing & editing, Visualization, Supervision, Investigation. **Kishanth Rameshkumar**: Methodology, extraction, Formal analysis, Validation, cell line studies ,Writing– original draft.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.


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## Ethics approval and consent to participate

Not applicable.

## Consent for publication:

Not applicable.

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