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Extraction and Evaluation of Collagen as Biomaterial from Chicken Shank

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ABSTRACT

Background: Collagen is the most abundant protein found mainly in skin, bone, tendon and cartilage of animals and humans. Due to biocompatibility, biodegradability and minimal immunogenicity, collagen is extensively used in biomedical applications such as wound healing, tissue engineering and drug delivery systems. The study was aimed at extracting the collagen from chicken shank and evaluate it morphologically to ascertain its purity for biomedical application.

Methods: A total of 24, day-old broiler chicks were procured from commercial poultry farm located at Varanasi, Uttar Pradesh. The chicks were reared at Livestock Farm Complex, Faculty of Veterinary and Animal Sciences, RGSC, Banaras Hindu University. The chicks were divided into three groups of 8 birds each, *viz.* Group 1, Group 2 and Group 3 sacrificed for extraction of collagen from their shank at 28, 35 and 42 days respectively. Chicken shanks collected were cleaned, trimmed, peeled, deboned and ground for further extraction process. The ground material was subjected to pre-treatment by alkali and acid followed by enzymatic hydrolysis using pepsin enzyme. Thereafter, pre-treated tissue from shank was subjected to precipitation, centrifugation, dialysis and finally lyophilization for freeze drying. The yield of shank extracted collagen was recorded on wet weight basis and dry weight basis. The morphological characteristics of the extracted collagen were evaluated by Hematoxylin and Eosin (H and E) staining, Masson's trichrome staining and electron microscopy.

Result: The yield of extracted lyophilized collagen from group 1, 2 and 3 on dry weight basis is recorded as 0.132%, 0.182% and 0.215% respectively; whereas, the yield of extracted lyophilized collagen from group 1, 2 and 3 on wet weight basis was recorded as 0.130%, 0.181% and 0.213% respectively. The extracted collagen samples exhibited off-white colour with spongy paper-like consistency. Microscopically, the collagen from group 1 was found more fibrous with sheet-like structures, collagen from group 2 exhibited relatively less spongy admixture of sheet and little fibrillar structures; whereas, collagen from group 3 revealed dense fibrillar structure. The minimal basophilic content and clear background of H and E stained sections indicated purity of extracted collagen. The study successfully documented the extraction of high-quality collagen suitable for various biomedical applications. In addition, the study underlined the potential of utilizing low-value chicken by-products as a sustainable and cost-effective source of collagen, offering both environmental and economic benefits.

Key words: Collagen, Morphology, Poultry, Shank.

INTRODUCTION

Collagen is the type of fibrous protein that is found in the skin, bones, tendon, ligaments and connective tissues of animals as well as humans. The primary role of collagen is to maintain tissue integrity, support cellular structure and promote elasticity throughout the body. Collagen molecules form three polypeptide chains and bind together in a triple helix. Each chain contains about 1000 amino acid residues in size and has an average length of 300 nm and a diameter of 1.4 nm. The various types of collagen found in nature are type I collagen predominantly present in skin, bones and connective tissues; type II collagen found in cartilage and type III collagen found in reticular fibers. Moreover, collagen is found as 28 types and each serves its distinct structure and properties (Cao *et al.*, 2020; Halson *et al.*, 2023). Collagen has a role in the biological functions of cells such as cell survival, proliferation and differentiation. It possesses unique properties such as biocompatibility, biodegradability and low immunogenicity, making it highly desirable for a wide range of biomedical applications. In this context, a wide range of studies are available in which collagen is used as scaffold in wound healing and bone grafting such as use of collagen

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membrane with bone graft for early bone healing with high osteoconductive support and early resorption (Preethi *et al.*, 2020). In addition Jain *et al*. (2023) concluded that titanium elastic nail as implant with Hydroxyapatite-collagen graft are compatible to the animal's body without any adverse effect.

The reports on extraction of collagen from chicken waste byproducts such as skin, bone, cartilage, feathers, shank *etc.* are meager as most of the studies targeting collagen have been done on large animals to maximize the yield. Chicken waste byproducts contain valuable collagen, which can be extracted and utilized in various industries including food, pharmaceuticals, cosmetics and biomedical applications (Gojkovic *et al.*, 2014). In addition, there are no social taboos and acceptability issues with poultry collagen like collagen extracted from large animals. Therefore, present research work is planned to extract the collagen from chicken shank and evaluate it morphologically to ascertain its purity for biomedical application.

MATERIALS AND METHODS

Sample collection

In the present study a total of 24, day-old broiler chicks were procured from commercial poultry farm located at Varanasi, Uttar Pradesh. Birds were reared at Livestock Farm Complex, Faculty of Veterinary and Animal Sciences, RGSC, Banaras Hindu University under standard management conditions. The study on broiler was approved by the Institutional Animal Ethics Committee (IAEC) with issued IAEC No. IAEC/RGSC-BHU/2023-24/180 and carried out in the Department of Veterinary Anatomy, FVAS, RGSC, BHU during January 2023 to October 2024. Birds were divided into three groups of 8 birds each *viz.* Group 1, Group 2 and Group 3 and were sacrificed for extraction of collagen from shank at 28, 35 and 42 days respectively as most commercial broilers reach slaughter weight between 4-6 weeks of age. The shank from all the sacrificed birds were collected and stored at -18±2°C in high density polyethylene (HDPE) bags till further processing.

Processing of collected shank for collagen extraction

The chicken shanks collected were cleaned, trimmed, peeled and deboned before mincing and grinding (Fig 1). The entire deboned chicken shank meat sample was kept in a freezer at -18±2°C in high density polyethylene (HDPE) bags till further processing. The ground material was subjected to different dry rendering temperature for removal of fat. The rendered fat *i.e.* liquid phase of low density was separated by squeezing with the help of muslin cloth and further separated from the high-density liquid phase with the help of a high-speed centrifuge machine at 5000 rpm for 10 minutes.

Proximate analysis of shank samples

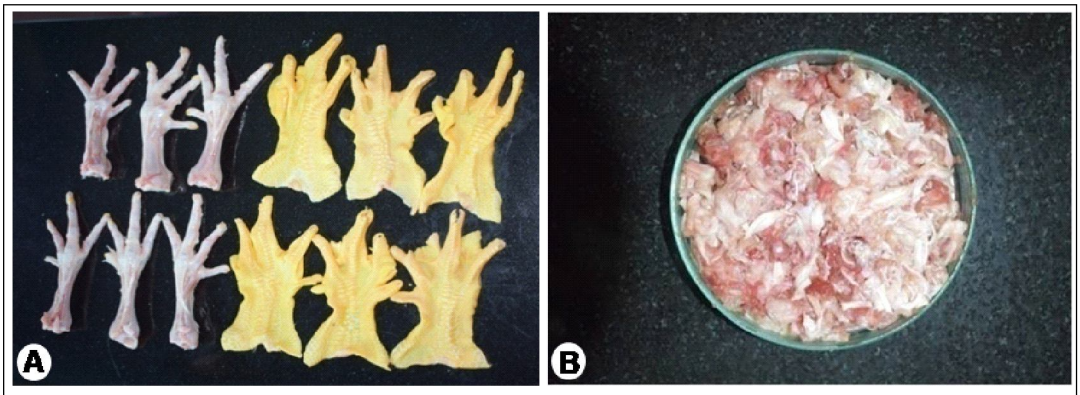
The fat, protein, ash and moisture content of the deboned shank was evaluated by proximate analysis to determine the water and protein levels, effectiveness of fat removal and understand mineral content; crucial for optimizing the collagen extraction process.

Fat content

The shank samples were subjected to extraction of fat using Soxhlet extraction assembly, as per the AOAC official method 948.22.1995. The oil flask and reflux condenser of the Soxhlet extractor was pre-dried and weighed. Sample (5 g) was placed in a thimble and dried at 95-100°C for 6 h in a hot air oven. Petroleum ether (150 mL) was poured into the flask as a fat extraction solvent and the condenser apparatus was set up over an electric heater to gently boil the solvent away. The extraction process was carried out for 16 hours followed by oven drying for evaporation of petroleum ether. The equation for fat estimation was mathematically expressed as:

$$\text{Fat (\%)} = \frac{W2 - W1}{W3} \times 100$$

Where,
W1 = Weight of empty oil flask.
W2 = Sum of weight of oil flask and fat.
W3 = Weight of sample taken.



A: Cleaned, trimmed and peeled shank along with skin; B: Deboned shank before mincing and grinding.

Fig 1: Processing of chicken shank sample.

Protein content

A pinch of digestion mixture (K_2SO_4: $CuSO_4$: 9:1) was added to the sample (1 g) to catalyze the digestion with 40 mL concentrated sulfuric acid in a Kjeldahl's digestion flask. The flask was heated gently in a tilted position till the frothing stopped followed by rapid boiling until the solution cleared. The solution was then allowed to cool to room temperature. The final volume of the solution was made up to 250 mL with subsequent addition of distilled water. Distillation of the mentioned diluted sample (25 mL) was carried out with the help of 40 mL NaOH solution (40% concentration), employing Kjeldahl distillation assembly. Steam was distilled with 40 mL of 4% boric acid (H_3BO_3) containing 8 drops of blended indicator dye (equal parts of 0.5% bromocresol green and 0.1% methyl red) for 5-10 minutes. The amount of trapped ammonia in boric acid was estimated by titrating against sulfuric acid (0.1 N). The percentage of nitrogen concentration was evaluated using the given formula:

$$\text{Nitrogen (\%)} = \frac{V \times 0.0014 \times \text{Total volume of digested sample made}}{\text{Weight of sample taken} \times \text{Volume of aloquot taken}}$$

Where,

V = Amount of acid consumed (N/10 Sulfuric acid) in mL.

The percentage of protein was calculated by converting nitrogen percentage into protein with a conversion factor of 6.25, presuming that all nitrogen in poultry skin was available as protein *i.e.*

$$\text{Protein (\%)} = \text{Nitrogen (\%)} \times 6.25$$

Ash content

For determination of total ash (AOAC, 1995), the fresh minced sample of 2 g was placed in a pre-weighed crucible and transferred to a muffle furnace at 600°C for 8 hours. The ash collected from sample was weighted with the help of weighing machine. The ash content was calculated by the following formula:

$$\text{Total ash (\%)} = \frac{W2 - W1}{W3} \times 100$$

Where,

W1 = Sum of weight of the crucible and sample before making into ash.

W2 = Sum of weight of the crucible and ash after making the ash.

W3 = Weight of sample taken.

Moisture content

Mashed sample (10 g) was transferred in a pre-weighed flat bottom aluminium moisture cup, which was transferred to a hot air oven at 100±1°C and kept for 24 hours. The dried sample was weighted with the help of weighing machine. Moisture content was calculated by applying the following formula:

$$\text{Moisture (\%)} = \frac{W2 - W1}{W3 - W1} \times 100$$

Where,

W1 = Weight of the empty cup.

W2 = Sum of weight of cup and sample.

W3 = Weight of cup and dried sample.

Extraction of collagen

The collagen extraction from rendered shank was performed according to standard methods with few modifications, in three major steps *viz.*, pre-treatment of the partially defatted rendered shank (Kittiphattanabawon *et al.*, 2005; Munasinghe *et al.*, 2014), collagen extraction from pre-treated shank (Munasinghe *et al.*, 2014) and precipitation along with dialysis of extracted shank collagen. The major steps involved in the extraction process are mentioned below.

Pre-treatments of dry rendered partially defatted shank

Alkali pre-treatment

Rendered shank was soaked in the 0.1 mol/L NaOH solution for 6 hours in a 1:10 ratio (w/v) at a temperature of 4°C and shaken at a speed of 165 rpm in orbital shaker incubator (REMI RIS-24 Plus TFT) with replacement of NaOH solution at 2 hours intervals followed by washing with double distilled water (DDW) until neutralization.

Chemical de-fatting

The dry rendered mechanically defatted alkali pre-treated shank was further treated with 10% butyl alcohol in 1:10 ratio (w/v) at a temperature of 4°C for 12 hours and speed of 165 rpm in orbital shaker incubator replacing the 10% butyl alcohol solution at every 6 hours interval. At the end of the chemical de-fatting, the shank was washed thrice with DDW.

Demineralization

The chemically defatted and alkaline pre-treated shank was further treated with 0.1 N HCl in 1:6 ratio (w/v) for 24 hours at 165 rpm under 4°C in orbital shaker incubator. This led to the removal of inorganic constituents while promoting shank swelling. At the end of demineralization, shank was washed thrice with DDW.

Collagen extraction

The pre-treated shank was used for extraction of collagen using pepsin in acetic acid. The process of extraction was optimized based on the yield of collagen.

a) Acetic acid treatment/Hydrolysis

In this method, rendered, alkaline pre-treated, chemically defatted and de-mineralized poultry shank was processed in a 1:6 ratio (w/v) of 0.5 mol/L acetic acid solution for 48 hours at 4°C and 165 rpm in orbital shaker incubator.

b) Enzymatic hydrolysis

In this method, rendered, alkaline pre-treated, chemically defatted and de-mineralized shank was hydrolyzed in a 1:6 ration (w/v) of 0.5 mol/L acetic acid solution containing 0.1% pepsin (activity of 1,000.0 U/mg) (w/v) for 24 hours at 4°C and 165 rpm in orbital shaker incubator.

Precipitation and dialysis of extracted collagen

The hydrolyzed, soluble collagen from the shank was filtered by a double layered nylon plastic strainer. The filtered liquid was precipitated with the help of 2.6 mol/L NaCl solution in Tris (hydroxymethyl) aminomethane hydrochloride (0.05 M) at a pH of 7.0. The precipitated collagen was sedimented with the help of a high-speed centrifugation machine (Eppendorf centrifuge 5810R, Germany) at 12,000 rpm for 15 minutes at 4°C. The white precipitated collagen (Fig 2A) was subjected for dialysis against 20 volume of 0.1 M acetic acid for 12 hours, followed by DDW for another 24 hours with the change of dialysis solution at every 6 hours. Dialysis was undertaken using a dialysis membrane (HIMEDIA, Dialysis membrane-50) at 4°C and 175 rpm in orbital shaker incubator. The dialyzed shank collagen was finally lyophilized (-40°C, 100 mbar pressure, 16 hours) in a freeze dryer (MAC, MSW-137) and stored in a sealed container under room temperature (Fig 2B).

Yield of collagen

Yield of shank extracted collagen on wet weight basis was calculated according to the method described by Toniasso *et al.,* 2022 using the following formula:

$$\text{Yield (on wet weight basis)} = \frac{\text{Weight of the lyophilized collagen (g)}}{\text{Wet weight of sample (g)}} \times 100$$

Yield of shank extracted collagen on dry weight basis was calculated according to the method described by Hashim *et al.,* 2014 using the following formula:

$$\text{Yield (on dry weight basis)} = \frac{\text{Weight of the lyophilized collagen (g)}}{\text{Wet weight of sample (g) - Moisture content of sample}} \times 100$$

Morphological characteristics of extracted collagen

Haematoxylin and eosin staining (H and E) and Masson's trichrome staining along with Scanning electron microscopy were performed for evaluation of general histo-architecture of extracted collagen fibers.

Haematoxylin and eosin staining

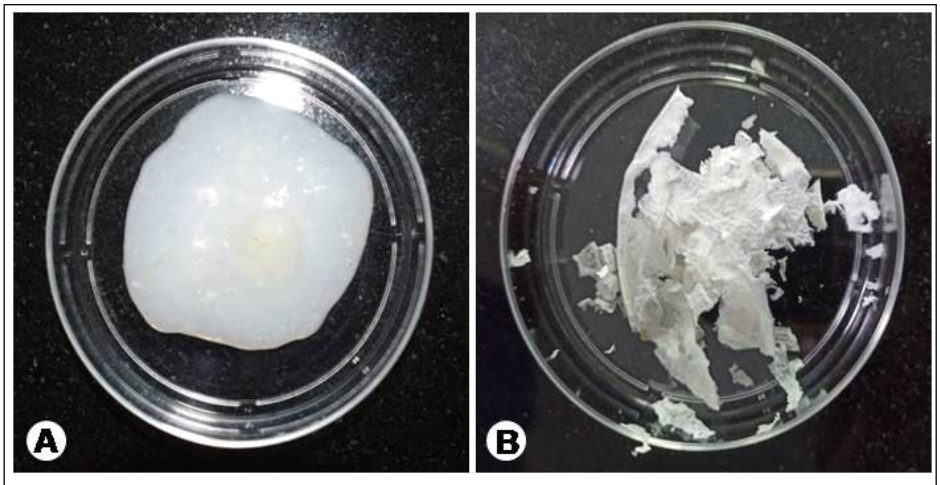
For histo-architecture studies, the thin pieces of formalin fixed extracted collagen were washed overnight under running tap water followed by dehydration through ascending grades of alcohol. After dehydration, clearing was performed using acetone and benzene followed by paraffin embedding by automatic tissue processor. Thereafter, 4-5 ìm thick paraffin embedded sections were trimmed by microtome and stained with routine haematoxylin and eosin stain using standard protocol (Bancroft and Gamble, 2008). Thereafter, the stained sections were examined under the microscope (Olympus CX21iLEDFS1, India) and photomicrography was performed.

Masson trichrome staining

For evaluation of morphology of collagen fibers, 4-5 µm thick paraffin embedded sections were trimmed by microtome and stained with Masson's trichrome stain using standard protocol (Bancroft and Gamble, 2008). Thereafter, the stained sections were examined under the microscope (Olympus CX21iLEDFS1, India) and photomicrography was performed.

Electron microscopy

Scanning electron microscopic studies were carried out in the Department of Physics, Institute of Science and Central Discovery Centre, Banaras Hindu University, Varanasi to see the surface details and organization of extracted collagen.



A: White coloured precipitated chicken shank collagen; B: Final lyophilized white coloured, spongy paper like chicken shank collagen.

Fig 2: Extracted chicken shank collagen.

RESULTS AND DISCUSSION

Weight of processed shank used for collagen extraction

The total weights of fresh shanks obtained from group 1, group 2 and group 3 were recorded as 310.08 g, 474.7 g and 487.9 g respectively. After peeling and removal of skin and nail, total weight of peeled shank from group 1, group 2 and group 3 were recorded as 197.35 g, 309.73 g and 321.8 g respectively. The weight of deboned chicken shanks was recorded as 61.27 g, 103.48 g and 114.6 g respectively. The weight of shank was highest in the group 3 and lowest in the group 1 suggesting that the weight of shanks increases as the age advances.

Proximate analysis of different groups of shank samples

The proximate composition of the broiler chicken shank samples is presented below in Table 1.

The percentage of fat in shank shows a significant role in the isolation and purity of collagen. Study by Potti *et al.* (2017) indicated that shank typically contain 3.90% fat. In addition, Hashim *et al.* (2014) analyzed the total crude fat in chicken feet is 3.90%. In contrary to this, in our study fat per cent in shank (without rendered) of group 1, 2 and 3 was found to be 9.0%, 10.60% and 14.80% respectively which may be the effect of peeling off and deboning. The fat content was highest in the group 3 suggesting more accumulation of fat in the older birds included in our study. The finding of Liu *et al.* (2001) is in line with our findings who reported the fat content in the range of 10-15%.

The protein content in groups 1, 2 and 3 was recorded as 22.75%, 19.90% and 17.50% respectively. The protein content is highest in group 1 suggesting the decrease in the protein content of shank with the age in our study. These findings are in line with the report of Potti *et al.* (2017) who found the protein content of approximately 18.10% in chicken feet.

The ash content in group 1, 2 and 3 was recorded as 4.28%, 2.40% and 2.46% respectively which indicates that the ash content of shank at different age groups ranges between 2%-5%. These findings are in line with the report of Liu *et al.* (2001) who reported similar ash content levels, indicating that our finding is consistent with the natural mineral content of chicken feet.

The moisture content in groups 1, 2 and 3 was recorded as 68.83%, 66.89%, 66.04% which generally ranges from 65% to 70%. This aligns with the study of Liu *et al.* (2001) who reported a moisture content of about 63% in chicken feet.

Table 1: Proximate composition of shank samples from different groups.

Parameters	Group 1 (28 days)	Group 2 (35 days)	Group 3 (42 days)
Fat (%)	9.0	10.60	14.80
Protein (%)	22.75	19.90	17.50
Ash (%)	4.28	2.40	2.46
Moisture (%)	68.83	66.89	66.04

Yield of extracted collagen

The final lyophilized collagen extracted from shank samples of groups 1, 2 and 3 was recorded as 0.08 g, 0.188 g and 0.245 g respectively. The yield of extracted lyophilized collagen from groups 1, 2 and 3 on dry weight basis is recorded as 0.132%, 0.182% and 0.215% respectively (Table 2); whereas, the yield of extracted lyophilized collagen from group 1, 2 and 3 on wet weight basis was recorded as 0.130%, 0.181% and 0.213% respectively (Table 2). The collagen content was found maximum in group 3 and minimum in group 1, suggesting the increase in collagen content with the age till 42 days.

The yield of collagen in our study was found to be significantly lower possibly due to peeling and deboning as the skin and bones contains higher amount of collagen (Munasinghe *et al.*, 2014). In addition, Suparno and Prasetyo (2019) shows that the difference in yield generally attributed to differences in the extraction method used, the concentration of the solution used to remove non-collagen protein, the type of raw material used, temperature differences and the duration of extraction process. In this context, we can conclude that the amount of collagen wasted during the pre-treatment and washing process may significantly decrease the yield. The shank has a dense or less porous physical form which causes a low level of swelling, so that absorption of water is low and the ionization reaction with water during the extraction process is reduced. Additionally, shank has a thick structure that requires extracting material that can penetrate into it aggressively. The small number of cross links opened during the swelling process can further reduce yield.

Morphological characteristics of extracted collagen

Macroscopic observation

Macroscopically, the shank extracted collagen from all the groups revealed white to off-white colour with slightly spongy, paper like consistency without clumps or irregularities. Similar findings were recorded in chicken feet collagen extracted using salt, acetic acid and pepsin in acetic acid by Zhou *et al.* (2016). Grossly, there was no

Table 2: Yield of extracted collagen based on wet weight and dry weight in different age groups of chicken shank.

Group	Age (in days)	Yield of collagen (on dry weight basis in %)	Yield of collagen (on wet weight basis in %)
1	28	0.132	0.130
2	35	0.182	0.181
3	42	0.215	0.213

difference in collagen extracted from different groups included in the study.

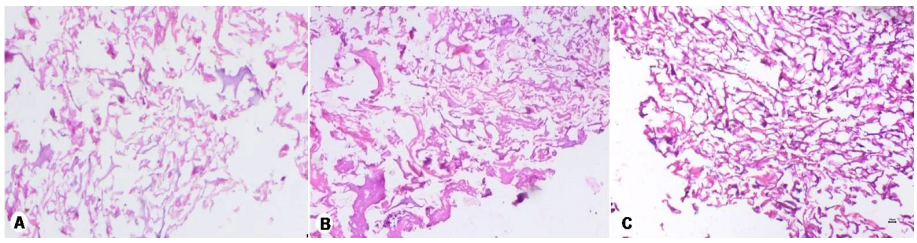
Microscopic observation

Hematoxylin and eosin staining

Hematoxylin and Eosin (H and E) stained smears typically revealed the presence of sponge like, wavy bundles of collagen fibers, which stained pink or dark pink in colour suggesting its proteinaceous nature. The density of these wavy bundles was recorded minimum in group 1 (Fig 3A) followed by group 2 (Fig 3B) and maximum in group 3 (Fig 3C) suggesting increase in the density of fibers with the age. Minimal to no blue or purple staining is observed indicating that there is minute to no cellular debris, such as nuclei. The background of the stained sample appeared relatively clear, further confirming the purity of the extracted collagen. The H and E staining indicated the successful extraction of collagen with high purity from shank sample.

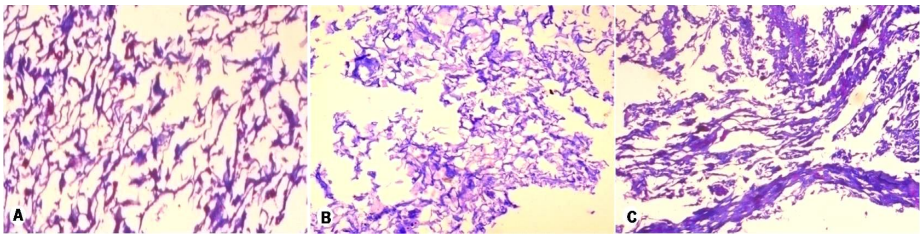
Masson's trichrome staining

The collagen fibers were prominently stained blue, displaying a dense and uniformly distributed interwoven structure, characteristic of well-preserved collagen (Fig 4).



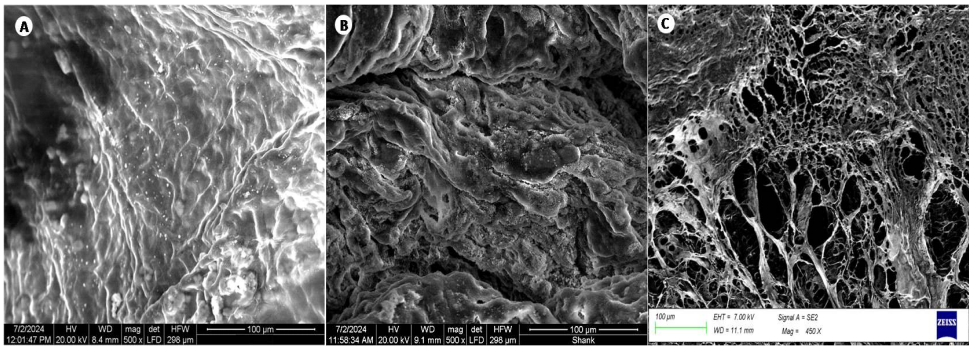
A: Presence of eosinophilic sponge like, wavy bundles of collagen fibers in group I. Collagen. H and E, 100X; B: Presence of eosinophilic slightly dense sponge like, wavy bundles of collagen fibers in group II. Collagen. H and E, 100X; C: Presence of eosinophilic web like, dense fibrillar collagen fibers in group III. Collagen. H and E, 100X.

Fig 3: Microphotograph of haematoxylin and eosin stained sections of extracted collagen.



A: Presence of bluish sponge like, wavy bundles of collagen fibers in group I. Collagen. Masson's trichrome, 100X; B: Presence of bluish slightly dense sponge like, wavy bundles of collagen fibers in group II. Collagen. Masson's trichrome, 100X; C: Presence of bluish dense fibrillar collagen fibers in group III. Collagen. Masson's trichrome, 100X.

Fig 4: Microphotograph of masson's trichrome stained sections of extracted collagen.



A: Presence of a sheet like collagen with minimum intertwined thin fibers of collagen in group I. Collagen. SEM, 500X; B: Presence of wave like, interwoven bundles of collagen fibers in group II. Collagen. SEM, 500X; C: Presence of dense mesh like fibrillar collagen fibers with characteristic visible lines suggesting the presence of the triple-helix structure in group III. Collagen. SEM, 450X.

Fig 5: Scanning electron microscopy (SEM) of extracted collagen.

In addition, presence of porous, sponge like, wavy bundles of collagen fibers in group I (Fig 4A), slightly dense sponge like, wavy bundles of collagen fibers in group II (Fig 4B) and dense fibrillar collagen fibers in group III (Fig 4C) were observed. The maximum thickness of collagen fibers was recorded in group 3 whereas collagen extracted from group 1 exhibited small interwoven fine fibers suggesting the increase in density and fibrillar structure with the age. The examination of Masson's Trichrome stained sections of extracted collagen indicated a high-quality extraction.

The process involved in this study effectively extracted collagen with high purity, ensuring that most other cellular components were removed. The clear visualization of collagen fibers with minimal extraneous tissue elements further confirms the success of the extraction method.

Electron microscopy

The scanning electron microscopy (SEM) results of extracted collagen from shank revealed detailed structural characteristics. At a broader 500 micrometer scale, the SEM images provided a comprehensive overview of the collagen's macrostructure, revealing a complex network of elongated, intertwined fibers. In addition, the SEM images showed a dense mesh of long, thin collagen fibers with characteristic visible lines suggesting the presence of the triple-helix structure (Fig 5). Presence of a sheet like collagen with minimum intertwined thin fibers of collagen in group I (Fig 5A), wave like, interwoven bundles of collagen fibers in group II (Fig 5B) and dense mesh like fibrillar collagen fibers with characteristic visible lines is observed in group III (Fig 5C).

The fibers exhibited a uniform and consistent surface morphology, confirming the high purity of the extracted collagen with preservation of its primary morphology. Studies by Wakjira *et al*. (2022) and Win *et al*. (2021) have shown that acid extraction methods, particularly using acetic acid, preserve the native fibrillar structure of collagen which is in line with our study as evidenced by SEM images that displayed well-organized and uniform fibers. The preservation of morphology is crucial for biomedical applications where the structural integrity of collagen matrices supports cell growth and tissue engineering. In this context, use of acid extraction method is of great importance as it maintains the desirable fibrillar network for precision applications. Although, the characteristic banding patterns of collagen are less prominent at this lower magnification, the overall uniformity and consistency of the fiber network remains evident.

CONCLUSION

The present study demonstrated the extraction of collagen from chicken waste byproducts as an alternative source for biomedical application. The extraction process involved multistep chemical and enzymatic treatment such as NaOH treatment, defatting with butyl alcohol, chemical treatments with HCl, acetic acid and pepsin followed by lyophilization to obtain the final product. The yield of collagen increased with the age indicating that older chickens store more collagen in their shank than very young chicks. In addition, very old chicken store less collagen as the synthesis of collagen is reduced.

The extracted collagen exhibited desirable morphological properties with no cellular and chemical debris, making it suitable for various biomedical applications. The findings underlined the potential of utilizing low-value chicken by-products as a sustainable and cost-effective source of collagen, offering both environmental and economic benefits. The success of this method opens new avenues for future research, including refining the process of collagen extraction to enhance yield and purity, scaling up production and exploring the creation of collagen films or powders for various applications.

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Ethics approval

The experimental work was approved by the Institutional Animal Ethics Committee (IAEC) with IAEC No. IAEC/RGSC-BHU/2023-24/180.

Disclaimers

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Informed consent

All animal procedures for experiments were approved by the Institutional Animal Ethics Committee (IAEC) with IAEC No. IAEC/RGSC-BHU/2023-24/180.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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