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Physico-chemical Characteristics of Keratin Extracted from Three Commercial Bird Feathers and their Antibacterial Activity

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Commercial bird feathers waste is a major environmental concern that must be modified in an eco-friendly way into value added products. In this study, keratin samples extracted from three commercial bird feathers (broiler, Turkey and country chicken) were compared and characterized. The chemically extracted keratin samples were analyzed using Kjeldahl method, SDS-PAGE, HPLC, SEM, FTIR, XRD techniques and also evaluated its antibacterial activity. The extracted samples showed low molecular weight and confirmed its secondary structure by FTIR spectra. The amino acids composition was similar in all keratin samples whereas the amino acids content was found to be higher in broiler keratin. All the samples retain their chemical structure and crystallinity. The antibacterial activity of keratin samples was identified by a well diffusion method and the zone of inhibition was found to be greater for broiler keratin compared to other samples. Thus, broiler feathers keratin can be utilized in future for more protein derived products due to its higher protein content and antibacterial activity.

Keywords: Chicken feathers, Precipitation, Keratin protein, SDS-PAGE, HPLC analysis.

INTRODUCTION

Nature presents a large or excessive amount of biological materials that have developed gradually for millions of years to have excellent properties and functions. Nowadays scientists were in search of novel effective eco-friendly materials from biological sources in order to minimize (or) remove the toxic effect of synthetic chemical material and also to improve its efficacy [1]. In recent years, biomaterials were developed rapidly in the medical field in order to augment/treat the disorder, diseases (or) trauma in an effective way. In this regard, the growth of biomaterials has drawn attention of many researchers to get new value added (or) therapeutic products [2]. Several protein-based biomaterials like collagen, gelatin, fibrin, silk and keratin have been investigated in the development of naturally derived biomaterials. Of these proteins, the use of keratin based biomaterials was reported in the past few decades due to their intrinsic biological properties [3].

Keratin is one of the most excellent biocompatibility, biodegradability and insoluble scleroproteins abundantly found in the body of mammals, reptiles and birds [4]. It is the predominant constituent of intermediate filaments of cytoskeleton and forms the bulk of the stratum corneum of the epidermis and epidermal appendages such as hair, wool, feathers, fingernails, animal claws and horn [5], which provide a shielding effect to the whole animal or to some critical parts of the body. It has been used extensively for various applications such as drug delivery, wound healing, tissue engineering, cosmetical, water purification, textile finishing and also act as composite materials in cell adhesion and proliferation process [6]. There are several instances of biological waste being disposed of in the environment, resulting in significant damage and disruption. Feathers are one such waste that cause environmental pollution, which is a regular, renewable and natural stuff produced in abundance. Approximately 5 million tonnes of feather biomass is being generated annually from poultry farms, which lead to serious solid waste hazards. The chicken feathers are the most troublesome waste product of the poultry industry, which contains 80-90% of proteins [7,8]. Keratin is a prominent complex protein present in the feathers. This protein is rich in cysteine residues which favours inter and intra molecular disulphide bonds. Due to this it exhibits properties like high toughness, high modulus,

high stability and high thermal resistance and make it insoluble in polar and non-polar solvents [9,10].

With regard to the secondary structure, keratin can be subdivided into α, β and γ-keratin (20), α-keratin (true keratin) protein are organized as spiral coils, mostly found in humans and the wool of other mammals in epithelial fiber cortex cells (50-60%), with molecular mass in the range of 40-60 Kd [11]. γ-Keratin are globular in nature with high content in glycine, cysteine and tyrosine which form the matrix in between the α-keratin filaments that constitute approximately 25% of the total protein [12]. It is a fibre with low molecular weight mass of around 15 Kd, act as disulfide cross-linkers that hold the α-keratin fibers together and give rise to the high mechanical strength, inertness and rigidity of the cortical support structure of hair and wool [13,14]. β-Keratin (corneous β-proteins) are found in feathers, beaks and scales of birds and reptiles, they protect the cortical filaments from chemical and physical damage and encoded by multiple genes [15], are located in tandem arrays on chromosomes 2, 25 and 27 in chicken. For β-keratin, the pleated sheet consists of laterally packed strands which can be parallel (or) anti-parallel; forming chains that are held together by intermolecular hydrogen bonds and peptide bond contribute to form a β-sheet, gives structural rigidity to the keratin protein [16]. Keratin is helpful to perform multiple haemostatic functions such as arresting bleeding and promote blood circulation and gives strength to epithelial cells against various types of mechanical and non-mechanical stress, including the maintenance of cellular integrity, regulation of cell growth, migration and protection from apoptosis. Keratin mutation or misregulation in the genes that encode keratin intermediate filaments causes several acute and chronic diseases in human skin and its appendages [17]. Keratin protein extracted from birds feathers exhibit great mechanical durability, bio compatibility and are easily biodegradable. These different properties of keratin have been used in the field of biotechnology, to characterize new keratin-based products such as hydrogel, films, fibers, sponges, with or without blended with other natural or synthetic polymers [18].

Huge amount of waste feathers are available from the activities such as shambles and animal rearing. In particular, the poultry industries generate huge amount of chicken feathers estimated worldwide nearly ay 8.5 million tons annually [19]. The effective and profitable use of this waste would both reduce the amount of harmful material in the environment and the consumption of resources. Moreover, the improper disposal of keratin waste contributes to environmental problems and disease transmission to the public. To solve this problem economically and environmentally, researchers are focusing to produce or design novel potential materials from keratin. A variety of methods are potentially available for extraction of keratin protein from birds' feathers such as enzymatic, chemical and with ionic solutions. The characteristics of keratin are determined by the method of extraction and its isoelectric point [20]. Recently, the keratin microparticles were synthesized from chicken feathers using chemical method and their characterization, antioxidant, anticancer and antimicrobial properties were studied [21-23]. Even so, there are still few literatures concerning the preparation and characterization of pure protein materials on keratin. Studies have reported in comparison and use of keratin extracted from human hair, sheep wool and chicken feathers to characterized for their chemical and conformational properties like amino acid composition, molecular weight, secondary structure and thermal properties [24]. In present study, keratin was extracted from different birds feathers using chemical method and the protein precipitated was collected using isoelectric point precipitation process. The objectives of this study was to characterize and compare the extracted keratin from different birds' feathers using SDS-PAGE, HPLC, FT-IR, XRD, SEM and antimicrobial property.

EXPERIMENTAL

Wet and fresh feathers of Turkey, broiler and country chicken were collected from slaughter house in Cuddalore, city, India. The chemicals used in this study, sodium sulfide, petroleum ether and HCl were purchased from Merck Ltd., India. All the chemicals used in the experiments were of analytical grade and distilled water was used for making solutions and washing.

Pre-treatment of feathers: Wet feathers were first washed in warm water (50 ºC) to remove blood, stains, dirt, oil and other impurities and dried in open air. The washed feathers were immersed in petroleum ether for 24 h for degreasing and then by washed with double distilled water. The washed feathers were conditioned at 30 ºC for 48 h at 70% relative humidity. Feathers were then cut into small pieces (1-2 cm), dried under sunlight for 48 h and stored at 4 ºC for further use.

Extraction of keratin: Keratin extraction was done according to the reported method [25] with slight modifications. Briefly, 25 g of pretreated chopped feathers of Turkey, broiler and country chicken were hydrolyzed separately with 0.5 M sodium sulfide solution at 50-60 ºC using mechanical stirrer for 6 h. The extracted hydrolyzed solution was filtered twice by using Grade 1 Whatman filter paper, to separate undissolved materials and centrifuged at 10,000 rpm to separate the supernatant. The filtrate obtained from Turkey, broiler and country chicken feathers showed the pH of 12.2, 11.6 and 12.4, respectively. The filtrate obtained was precipitated by adjusting the pH to 4.7 to the isoelectric point of keratin, using 2 N HCl. At pH 4.7, a thick layer of precipitate was settled down after 24 h, centrifuged at 12,000 rpm for 15 min, to remove the salts and other impurities. This process was repeated twice. Finally, the keratin sediment collected was dried at 45 ºC for 12 h and lyophilized to obtain keratin powder. The total protein content in the extracted keratin from Turkey, broiler and country chicken was calculated using Kjeldahl nitrogen determination method.

SDS-PAGE analysis: The electrophoretic separations of different feather keratins were performed on 15% (w/v) polyacrylamide separating gel and 5% (w/v) polyacrylamide stacking gel system. The keratin samples from three different bird feathers were dissolved in distilled water and boiled for 8 min with loading buffer that contains β-mercaptoethanol. The protein marker and denatured sample solutions were loaded onto the gradient polyacrylamide gel system. The electrophoretic separation was performed at 80 V for 30 min and followed by 120 V for 60 min. After that, the gels were rinsed twice with distilled

water before staining. After staining with Coomassie brilliant blue for 30 min, destaining was done overnight with destaining solution (ethanol-acetic acid) with orbital shaker. Finally, the gel image was taken with an imaging system.

Determination of protein content: Each keratin sample (100 mg) from different source was placed in a Kjeldahl flask, subsequently digested with 4 mL conc. H_2SO_4 containing a mixture of Na_2SO_4 and $CuSO_4$ (5:1) in the presence of N-catalyst. The digested solution was neutralized with 20 mL of 40% NaOH and distilled in 4% boric acid solution. Distillation was performed until the distillate volume was 60 mL. The borate anions formed was titrated with 0.5 M H_2SO_4, which then was converted to nitrogen in the sample. The volume of H_2SO_4 utilized to estimate the nitrogen concentration in the sample was calculated using the formulas:

$$N\ (\%) = \frac{\text{Titration volume (mL)} \times \text{Normalize } H_2SO_4\ (0.5\ N) \times \text{Nitrogen atoms mass}}{(14.008)\ \text{Sample mass (mg)}}$$

Protein concentration (%) = Nitrogen concentration (%) × Conversion factor (6.25)

Determination of amino acid composition: Free amino acids in keratin samples obtained from Turkey, broiler and country chicken feathers were post derivatized with orthophosphoric acid and analyzed by HPLC system (Shimadzu) equipped with a C-18 reverse phase column, flow rate 10mL/min, temperature 20 ºC and the eluate was detected at 338 nm. The keratin solution sample was 5mL used tetrahydrofuran as solvent for analysis. Individual amino acids concentrations were determined from the retention times and peak areas from standard amino acid mixture. The quantitative amino acid composition was expressed as% for each amino acid.

Characterization

Scanning electron microscopy (SEM): The surface of lyophilized keratin powder from broiler, country and Turkey feathers were studied under scanning electron microscopy (TSCAN) at an accelerating voltage of 10 kv.

Fourier transformed infrared spectroscopy (FTIR): The chemical structures of the keratin from Turkey, broiler and country chicken feathers were analyzed with a FT-IR spectrometer with a wavenumber range of 4000-500 cm^{-1}.

X-Ray diffraction (XRD): The X-ray diffraction study was carried out on keratin obtained from Turkey, broiler and country chicken feathers using an X-ray diffractometer (Bruker USA D8 Advance, Davinci model). Diffraction intensities were recorded in the 2 theta ranging from 10º-80º to determine the chemical changes (level of crystallinity) from keratin samples.

Antibacterial susceptibility test: Antibacterial sensitivity was done by agar well-diffusion method using Mueller-Hinton agar. The test bacterial strains used in this study were *Klebsiella*, *Staphylococcus aureus* and *Escherichia coli*. Overnight culture of *S. aureus*, *Klebsiella* and *E. coli* was inoculated into Muller-Hinton agar using sterile cotton swab. The wells of 6.0 mm in diameter were cut out on the seeded plates using sterile cork borer and each of the well was filled with the keratin samples from Turkey, broiler and country feathers of different concentration (2.5 µL, 5 µL, 7.5 µL and 10 µL) to the wells using micropipette. Then, the plates were incubated at 37 ºC for 24 h in upright position. It was incubated for 24 h at 37 ºC and zone of inhibitions were observed.

RESULTS AND DISCUSSION

Keratin is fibrous protein with high biodegradability, high sulphur content and nitrogen content, compared to other proteins. The protein and amino acids in keratin have become promising solution to enhance the material properties of biopolymers. Keratin are stabilized by disulphide cross linkages of cysteine, it must be split by during alkaline hydrolysis in order to extract the keratin. The extract keratin from feathers of Turkey, broiler and country chicken where tested for its protein content.

The dried hydrolyzed keratin sample of all three birds feathers contain 12.49, 13.55 and 11.56% of nitrogen respectively (Table-1). The differ in nitrogen content in different feathers may be due to the differing diets and metabolism of these birds. Among the three birds feather, broiler feather keratin showed increased nitrogen content compared to others. According to Kuncaka *et al.* [26], the higher in pigmentation in feathers cause reduction in the percentage nitrogen content as compared to the relatively unpigmented species. This correlates with the present findings that the percentage of nitrogen content in Turkey and country chicken feathers keratin showed reduction in nitrogen content due to that heavily pigmented feathers when compared with broiler chicken feathers keratin.

TABLE-1
TOTAL NITROGEN AND PROTEIN CONTENT IN TURKEY, BROILER AND NATIVE CHICKEN FEATHERS KERATIN (percentage/mg of protein)

Sample nature-solid	Turkey	Broiler	Country
Nitrogen	12.49	13.55	11.56
Protein	78.44	84.69	72.29

The protein content in feathers of Turkey was found to be 78.44% and that of broiler chicken feather 84.69% and country chicken feather 72.27% respectively. Among the three bird feathers broiler chicken feather showed high protein content due to their supplementation and growing conditions. The decreasing protein content in country chicken and Turkey feathers may be due to insufficient amount of essential amino acids in their diet and also cannot be synthesized by their own body [27]. For proper growth of bird the amino acids both essential and non-essential must be supplied in optimal ratio. If any limiting amino acids supplied in excess will not be used for the protein production, instead used as a source of energy after deamination. This conversion of excess limiting amino acids to nitrogen excretion products may leads to decreased protein content in country and Turkey feathers.

SDS-PAGE: The SDS-PAGE analysis of keratin extracted from three different birds feathers are shown in Fig. 1. The SDS-PAGE patterns show that the molecular weight values of the keratin in the three studied bird feathers were influenced by the extraction protocol. Sodium sulfide treatment produced prevalently low molecular weight keratins, confirming the degradation of the proteins caused by peptide hydrolysis at high pH

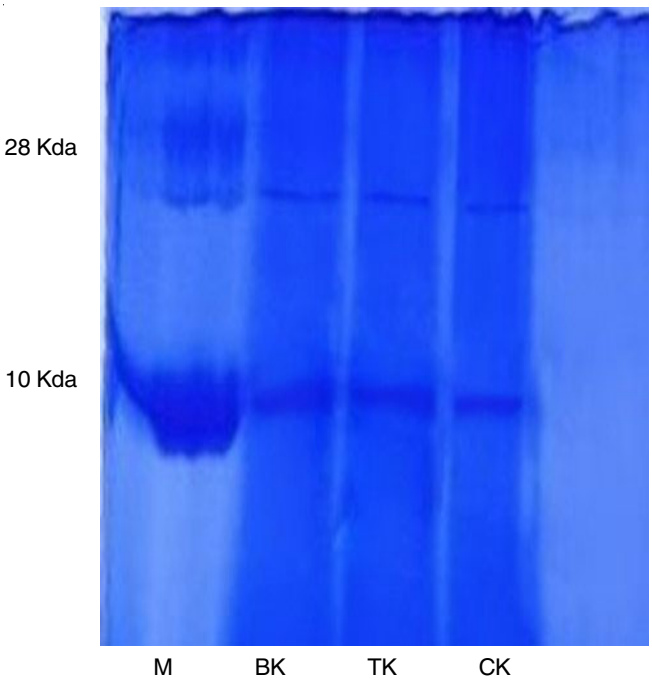

Fig. 1. SDS-PAGE gel of extracted keratin from broiler chicken feathers, Turkey feathers and country chicken feathers [Lane-M: Molecular weight of standard protein marker, Lane-1: broiler chicken feathers keratin (BK), Lane-2: Turkey feather keratin (NK), Lane-3: country feather keratin (TK)]

values [28]. The results showed two clear protein bands between 10 and 28 KDa, which are in agreements with the literature [29]. The protein bands obtaining from the three different bird feathers showed molecular weight of 10 and 28 KDa was mainly due to the β-keratin content of low molecular weight fraction.

Amino acid composition: HPLC system was applied to investigate the amino acids compositions in extracted keratin from Turkey, broiler and country feathers and the spectra are shown in Fig. 2. The amount of each amino acid present in keratin protein is shown in Table-2. The highest proportion of hydrophobic/hydrophilic amino acids was found in Turkey (47/29), followed by broiler (50/32) and country chicken (43/27) [30]. Glycine, serine, leucine, glutamic acids and alanine were the most abundant amino acids found in the three bird feathers. The extracted keratin showed the presence of 18 amino acids in different composition among the three samples. Understanding the nature of amino acids and it arrangement with in the protein molecule is essential to identify the change of the protein structure and its hydrophilic (or) hydrophilic nature. Due to their hydrophobic nature and presence of disulfide bonds due to the interaction of cystine makes them water-insoluble [31]. In this study, the keratin obtain from different feathers, the content of histidine, methionine and lysine in the protein of Turkey and country chicken feathers showed lower than the keratin extracted from broiler chicken feathers. These variations might be due to diet supplementation and various feeding habits showed less than 2% [32-34].

FTIR studies: The FTIR spectra of broiler, Turkey and country chicken feather are shown in Fig. 3. All the three spectra, the broad peaks around 3400 cm^{-1} was attributed due to the stretching vibration of O-H and N-H bands. The peaks around

TABLE-2
AMINO ACID CONTENT OF TURKEY FEATHERS KERATIN (TK), BROILER FEATHERS KERATIN (BK) AND COUNTRY FEATHERS KERATIN (CK) (Amino acid in protein %)

Amino acids	Contents (%)		
	Turkey feathers keratin	Broiler chicken feathers keratin	Country chicken feathers keratin
Aspartic acid	4.97	5.18	4.32
Glutamic acid	7.73	8.06	7.25
Histidine	0.76	0.87	0.67
Tyrosine	1.05	1.09	0.96
Methionine	0.82	0.86	0.77
Phenylalanine	5.59	5.72	5.11
Lysine	0.54	0.56	0.52
Leucine	6.97	8.31	6.36
Isoleucine	2.92	3.00	2.78
Arginine	3.61	3.97	3.47
Cysteine	3.37	3.46	3.14
Valine	4.72	5.89	4.51
Threonine	3.21	3.27	3.12
Alanine	6.52	6.71	5.45
Serine	7.84	9.29	7.25
Glycine	9.95	10.32	9.04
Proline	6.36	6.51	6.06
Asparagine	1.45	1.53	1.33
Total content	78.38	84.69	72.11

2945 cm^{-1} were due to the stretching vibration of C-H bands. The peaks present between 1680-1630 cm^{-1} confirmed the presence of amide-I components in the β-pleated confirmation of obtained protein. The peaks present between 1570 to 1515 cm^{-1} confirmed the presence of amide-II confirmation. The peak at 1534 cm^{-1} was due to the C-H stretching bonds present in amide-II confirmation, while the peak at 1658 cm^{-1} was due to the C=O stretching bond in the amide-I confirmation. The peak around 1400 cm^{-1} was due to the C-N stretching bonds and N-H bonding vibrations in the amide-III components of proteins. The peaks around 1230 cm^{-1} was due to the presence of C-N stretching bonds in amide-III components [35,36]. The obtained results confirmed that there are no significant changes in the spectral peaks of three bird feathers keratin after alkaline treatment. The absorbance spectra of all the tested bird keratin samples showed the similar pattern of characteristics peaks as reported earlier [34]. The isoelectric precipitation of keratin protein at pH 4.7 in broiler chicken feathers, Turkey feathers and country chicken feathers did not affect the chemical structure of protein significantly.

XRD-crystal structure analysis: The extracted keratin from different bird feathers showed peaks at 17.1º, 19.5º, 29.5º, 22.9º, 30.1º and 22.5º (Fig. 4). The peaks formed between 15º and 31º was due to the α-helical structure, where as the peaks formed in the range 17.32º, 21.80º, corresponds to the β-sheet protein structure. The strong peaks at 19.75º, 22.38º and 22.15º were indexed for β-sheet crystalline structure of keratin. The peak around 17.10º indexed for α-helix diffraction factor. All the samples found to contain more amount of β-sheet compare to α-helical structure and the results are similar to the literature [37,38]. The α-helical content is more in country chicken and Turkey feather keratin when compared with broiler keratin.

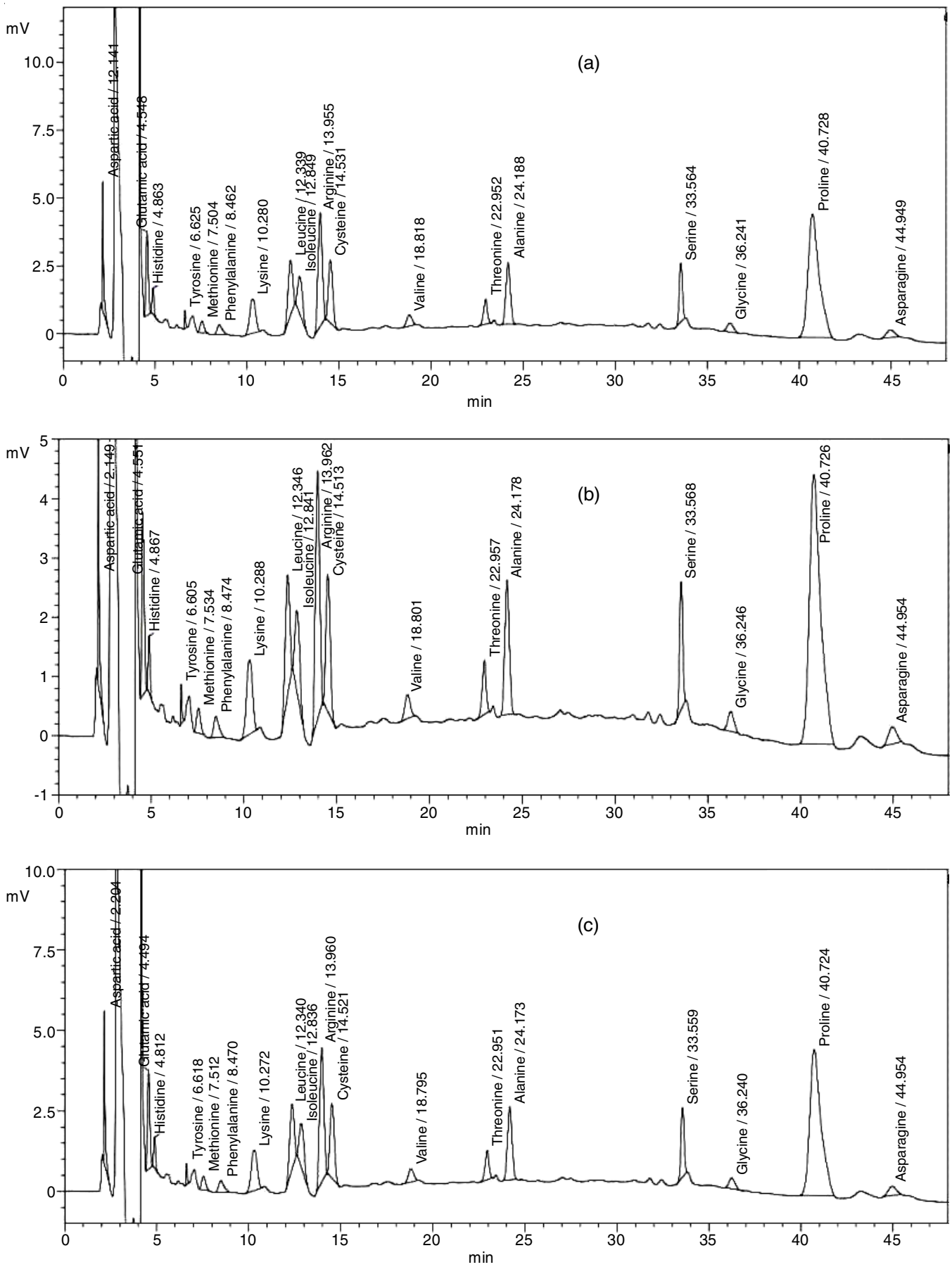

Fig. 2. Chromatogram showing the separation of amino acids of (a) Turkey, (b) broiler and (c) country chicken feather keratin

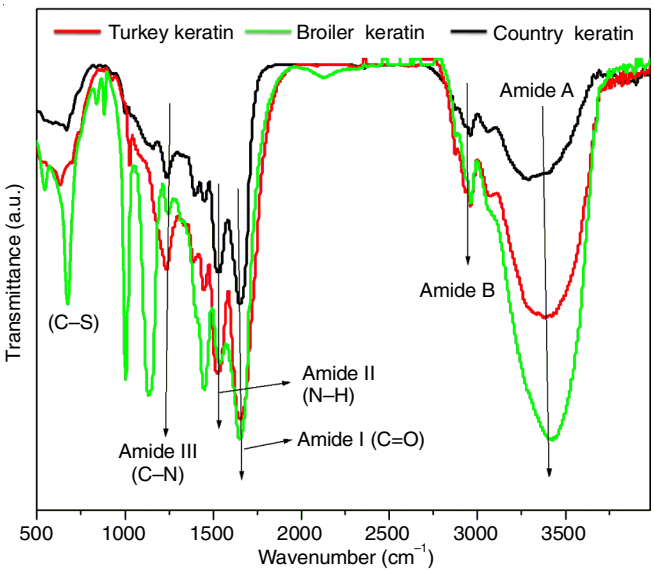

Fig. 3. FTIR spectra for three different keratin samples

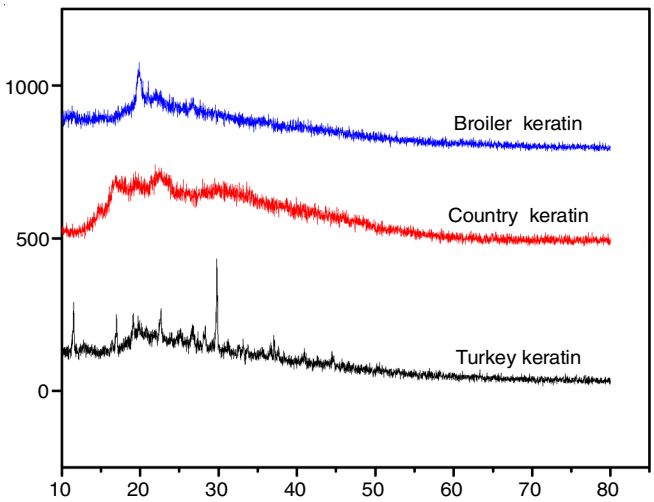

Fig. 4. X-ray diffractograms of three keratin samples

Surface morphology of keratin particles: The extracted keratin powder form broiler, Turkey and country feathers showed randomly arranged microsphere structure (Fig. 5), which are almost similar to the results of micrographs were show in the previous report of Singamneni *et al.* [39]. The micrographs also showed the smooth spongy microspherical particles and porous nature, which can be exploited for various biological applications.

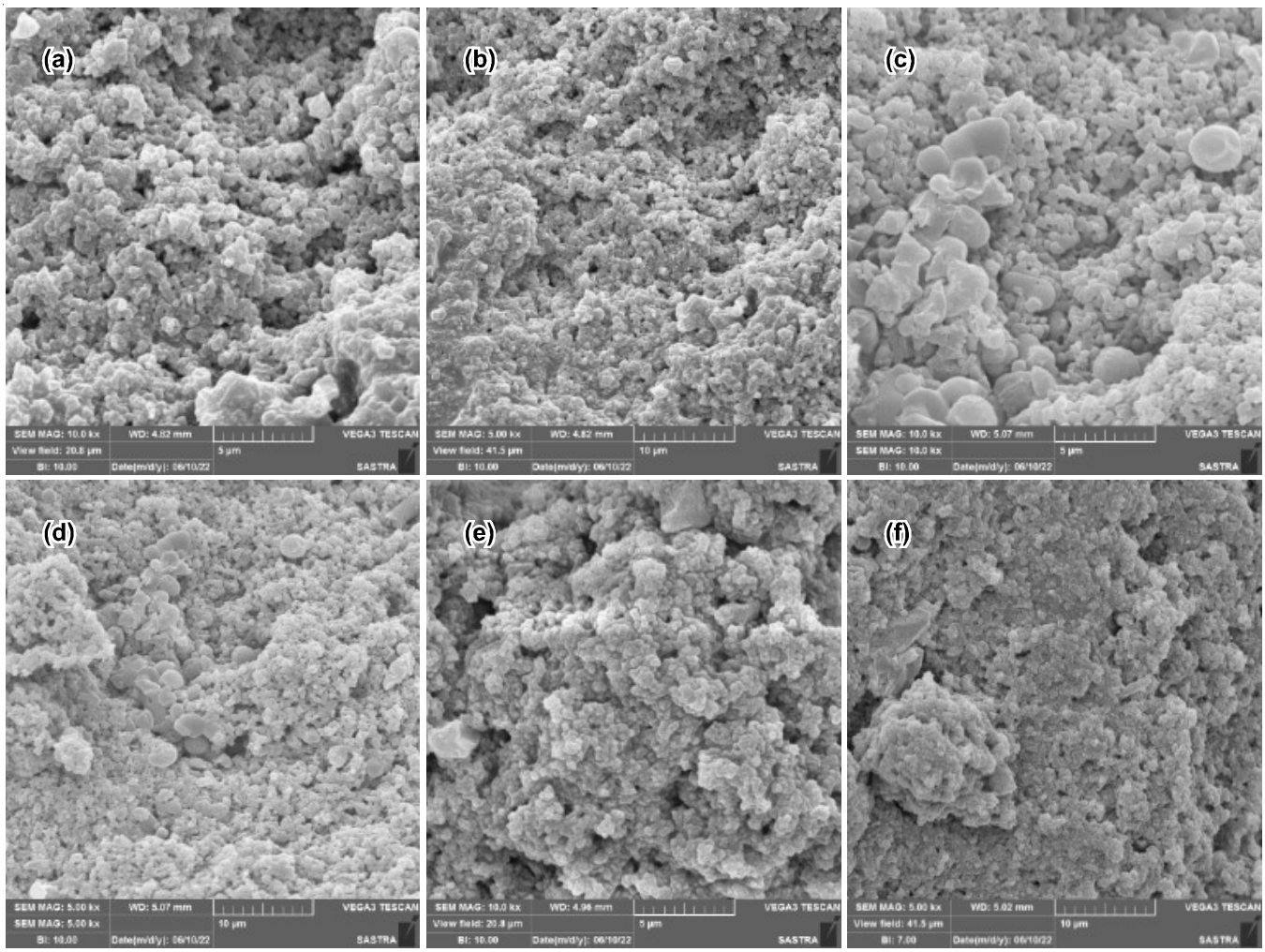

Fig. 5. SEM images showing morphology of TK-Turkey feather keratin (a&b), BK- broiler feather keratin (c&d) and CK-country feathers keratin (e&f)

Antibacterial activity: The antibacterial activities of the extracted keratin were assessed through agar diffusion methods. The antibacterial activity of keratin extracted from Turkey, broiler and country feathers was studied against *S. aureus*, *Klebsiella* and *E. coli*. The larger area of the zone indicates the higher ability of bacteria inhibition. The bacteria inhibition zone was observed and recorded with photographs (Fig. 6). The antibacterial activity in country chicken feather keratin and Turkey feather keratin shows less bacterial inhibition when compared with broiler chicken feathers keratin (Fig. 7). In BK (broiler keratin) sample exhibited strong antibacterial activity against Gram-positive and Gram-negative bacterial strains when compared with TK (Turkey keratin) and CK (country chicken keratin). In BK a high zone of inhibition (> 5 mm) was observed in *Klebsiella* than in *S. aureus,* indicated superior susceptibility towards Gram-negative bacteria. The TK and CK showed a less zone of inhibition against Gram-positive and Gram-negative bacteria. However TK possesses a positive antibacterial efficacy in *S. aureus, Klebsiella* and *E. coli*, when compared with CK but it was less zone of inhibition when compared with BK.

From the amino acids analysis, keratin extracted from the chicken feathers is found to be characteristically high in anti-oxidant amino acids such as glycine, cysteine, proline and

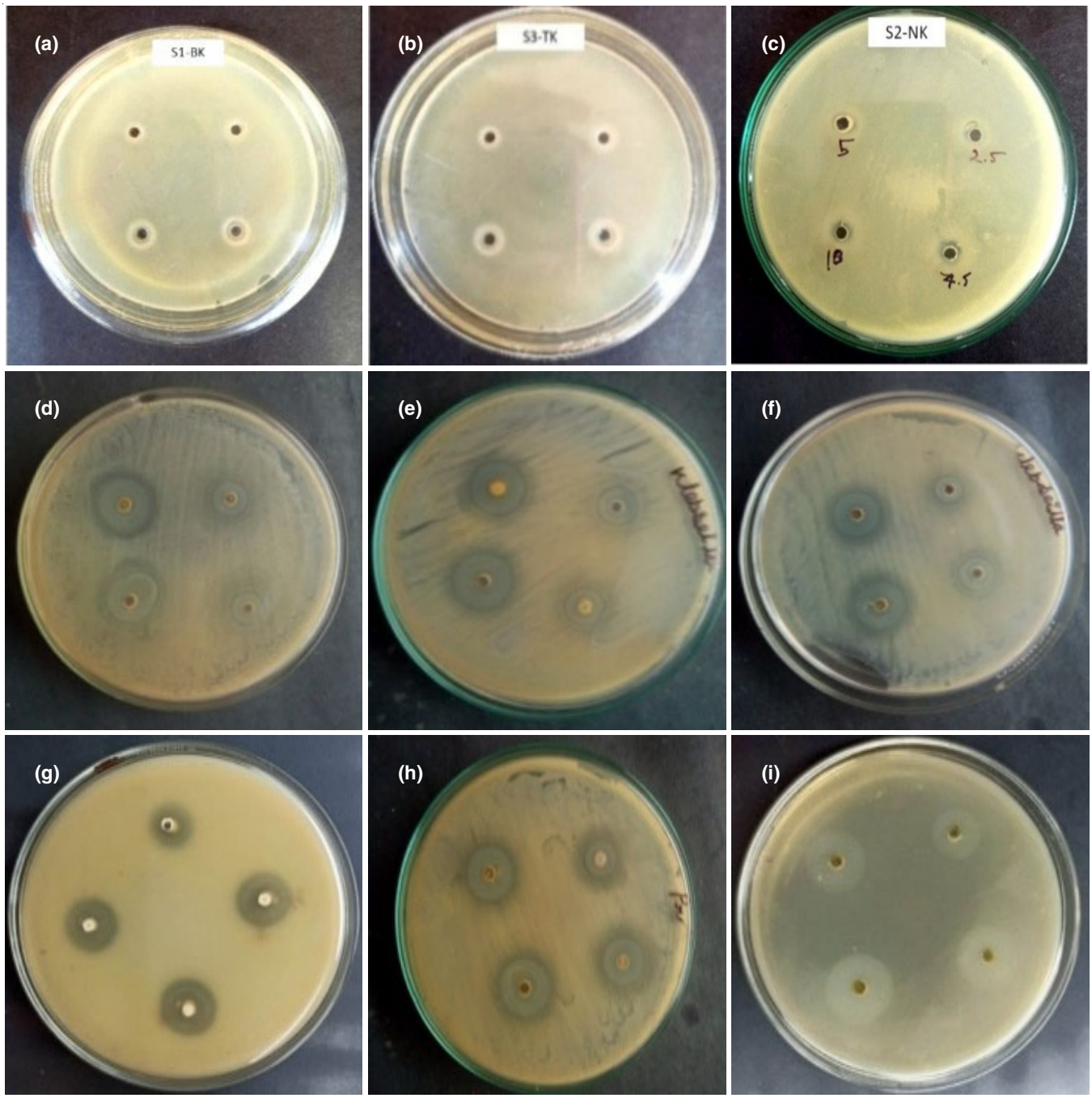

Fig. 6. Antibacterial activity of Turkey keratin (TK), broiler keratin (BK) and country chicken keratin (CK) against *Staphyococcus aureus* (a, b & c), *Klebsiella pneumoniae* (d, e & f) and *Escherichia coli* (g, h & i)

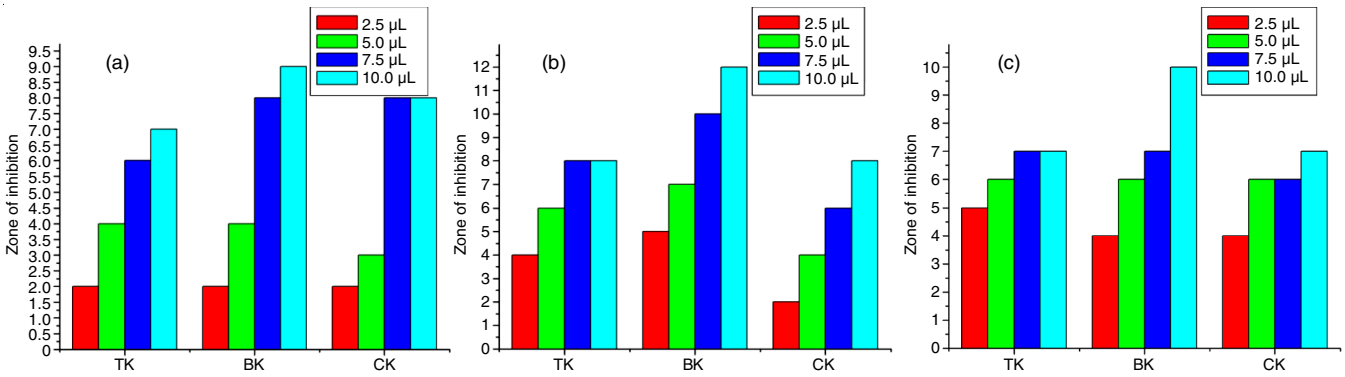

Fig. 7. Antibacterial activity of Turkey keratin (TK), broiler keratin (BK) and country chicken keratin (CK) against (a) *Staphylococcus aureus*, (b) *Klebsiella pneumoniae* and (c) *Escherichia coli*

lysine when compare with Turkey and country birds feathers. Previous study reported keratin protein from quail feathers exhibits antimicrobial activity against *S. aureus* and *E. coli* was due to that functional groups present in the keratin protein, especially peptide backbone, such as disulfide (S-S), amino (-NH_2) and carboxylic acid (-COOH) [40,41].

Conclusion

In this study, keratin was extracted from Turkey, broiler and country chicken feathers using sodium sulphite method and characterized. The protein content, amino acid composition, structural conformation and antibacterial properties of the three keratin samples were studied and compared. All the samples showed the presence of α and β-structures and exhibit low molecular weight. Among the samples broiler keratin exhibit high protein content, crystallinity and antibacterial property compared to other two samples. Moreover, the feather wastes output from broiler chicken was more significant when compared to other bird's feather. In spite of its waste volume, the keratin derived from broiler chicken displayed more protein content, crystallinity and antibacterial activities. These properties can be exploited by blending with other composites, to make it into functional materials that can be used for various applications like wound dressing, tissue engineering, drug delivery, *etc*.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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