PREPARATION AND CHARACTERIZATION OF CHICKEN FEET COLLAGEN FOR BIOMEDICAL APPLICATION

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Abstract

In this study, collagen was extracted from chicken feet and its biomedical application was investigated. The chicken feet were collected from poultry Myanmar market. The collagen was extracted from chicken feet by using acid solubilized method. The yield percent of crude was found to be 10.08 %. And then the crude collagen was purified by dialysis method, purified collagen was found to be 8.32 %. Both crude and purified collagens were characterized by using SEM, UV-visible and FT IR analysis. The SEM images of collagen to be regular and uniform with networking of porous on the surface**.** The absorption bands of crude and purified collagen were found to be near 230 nm in UV spectra. In the FT IR spectra, absorption bands of collagen sample indicated the presence of N-H, O-H, C=O, $-CH_2$, $-CH_3$ groups in the sample. The antimicrobial activity of both collagen samples were determined by agar well diffusion method. In this experiment, both samples were found to be high activities against six microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans and Escherichia coli*, which showed that collagen possessed the high antimicrobial activity against all tested microorganisms. The wound healing effect of both collagens was investigated. It was found that the purified collagen was well-developed in healing of burn wound with well-developed sebaceous glands, sweat glands and hair follicles in epidermis layer of skin. This research therefore contributes to academics as well as biomedical application.

Keyword: Chicken feet, collagen, acid-solubilized method, antimicrobial activity, biomedical

Introduction

Collagen was first found and defined as gelatin by extractive cooking of bones at the beginning of 1800's. This family of proteins was studied by scientists in different areas. Collagens are the most abundant high molecular weight proteins in both invertebrate and vertebrate organisms, including mammals and possess mainly a structural role, exciting different types according with their specific organization in distinct tissues (Almeida and Lannes, 2013). It is mainly presented in all connective tissues, including animal skins, bones, cartilages, tendon and blood vessels. It is involved in the formation of fibrillar and microfibrillar networks of the extracellular matrix and basement membranes. Collagen is the major components of the extracellular matrix nearly 28 types of collagen have been identified. All of them have triple helix characteristic but the length of the helix, the size and nature of non - helical portion various from one to another type (Kirti and Khora, 2015). The collagen types were classified by their size, function and distribution which differ considerably in their amino acid composition. Among all the current variants of collagens, type I, II, III and V are the most prevalent and they are all fibrillar-forming collagens. Every collagen type has its special amino acid composition, and each performs a distinctive role in the tissue (Hashim, 2015).

The collagen family consists of 28 different proteins, which account for 25% -35% of the total protein mass in mammals and play a pivotal role in the structure of several tissues, such as skin and bones, providing rigidity and integrity (Silvipriya, *et al*. 2015). The stability of collagen helix is closely correlated with the total amino acid content in a collagen molecule. Collagen contains rather larger amount of polar amino acid residues (arginine, lysine, aspartate, and glutamate) besides high contents of glycine and amino acids (Kiew and Don, 2013). The chemical

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cross – linkers used may be either relatively small bifunctional molecules or polyfunctional macromolecules. Poultry feet are abundant in collagen and also have been extracted as medical material such as collagen film, and collagen powder for wound exudate control (Lee and Singla, 2001).

Recently, consumers had rejected some foods and cosmetics that were prepared from beef collagen due to the fear of bovine spongiform encephalopathy. Thus, it is desirable to seek an alternative source of collagen from the other animal species rather than from cattle. Chicken feet may be a good collagen source and could be used to replace beef collagen. Collagen was extracted from different raw materials has been used for clarifying beverages, in cosmetics, in casings for meat products and in a host of biomedical application. Medical applications of collagen include use in drug delivery systems, sponges for burns and wounds and in tissue engineering (Liu, 2001).

Materials and Methods

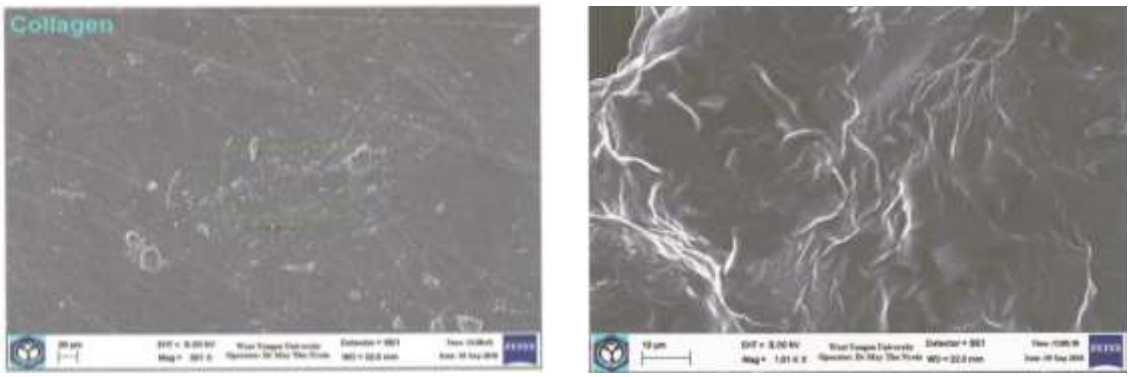
The chicken feet sample was collected from Hledan Market, Kamayut Township, Yangon. The chicken feet were cut proper size and washed with water to removed impurities. The collagen was extracted according to the method of Liu, 2001 and Lin, 2013 with slight modification. Repeated defatting processes were performed to extract lipids and triglycerides by stirring the chicken feet in 1:8 (w/v) of 90 % ethanol for 48 h at 4 ^{o}C. The residues were soaked with 0.05 M disodium salt of EDTA with a solid/ solvent ratio of 1:8 (w/v). The mixture was stirred for 24 h and then the solution was decanted and the residue was washed with distilled water, until the water was clear. The liquid suspension was decanted and the residues were subsequently soaked into 0.1 M NaOH at a sample / alkali solution ratio of 1: 8 (w/v) and stirred for 72 h at 4 ^{o}C then the liquid suspension was decanted and the residue was washed with distilled water, until the water was clear. Inorganic compounds were removed by soaking the defatted sample in 0.1 M HCl solution with solid/ solvent ratio of 1:8 (w/v). The mixture was stirred for 24 h. Then the solution was decanted and the residues were washed with distilled water until the neutral pH. After being wash with water, the residues were soaked in 0.5 M acetic acid with a sample per solvent ratio of 1:10 (w/v) at 4 ^{o}C for 5 days with a stirring. The chicken feet suspended solution was filtered by using cheese cloth to remove the residues. Then the solution was centrifuged at 15000 rpm for 30 min at 4 ^{o}C, the crude collagen solution was obtained. Crude collagen purification was achieved by convenient salting out process performed at 4 ^{o}C for 24 h with gentle agitation: NaCl was carefully added to the supernatants until a final concentration of 0.9 M was achieved. The collagen solutions were dialyzed against 0.1 M acetic acid and distilled water at 4 ^{o}C for 4 days and then dried at 30 ^{o}C for 3 days. And then the crude and purified collagens were characterized by SEM, UV, and FT IR. The antimicrobial activity of both collagen samples was determined by agar well diffusion method. The preliminary characterization and bioactivity test was made for biomedical application of collagens. The biomedical application of crude and purified collagens was further studied by animal test.

Results and Discussion

Chicken feet may be a good collagen source and could be used to replace beef collagen (Liu, 2001). There are three major methods of collagen extraction producer such as neutral-salt solubilized collagen, acid-solubilized collagen and pepsin-solubilized collagen. Organic acids are capable of solubilizing non-crosslink collagens and also of breaking some of the inter-strand cross-links in collagen, which leads to a higher solubility of collagen during the extraction process. Therefore, acidic solutions, especially acetic acid, are commonly used to extract collagen. In this study, collagen was extracted from chicken feet by acid solubilized method with acetic acid. The yield percent of crude and purified collagens were found to be 10.08 and 8.23 %, respectively.

Scanning Electron Microscopy (SEM)

Figure 1 (a) and 1 (b) show the surface morphology of crude and purified collagens. There were observed fibril like structures. The SEM images of collagen to be regular and uniform with networking of porous on the surface. Generally uniform network structure of collagen as drug carrier is propitious, for well-proportioned distribution of drugs. Based on the SEM results, chicken feet collagen is suitable for the preparation of collagen based products.

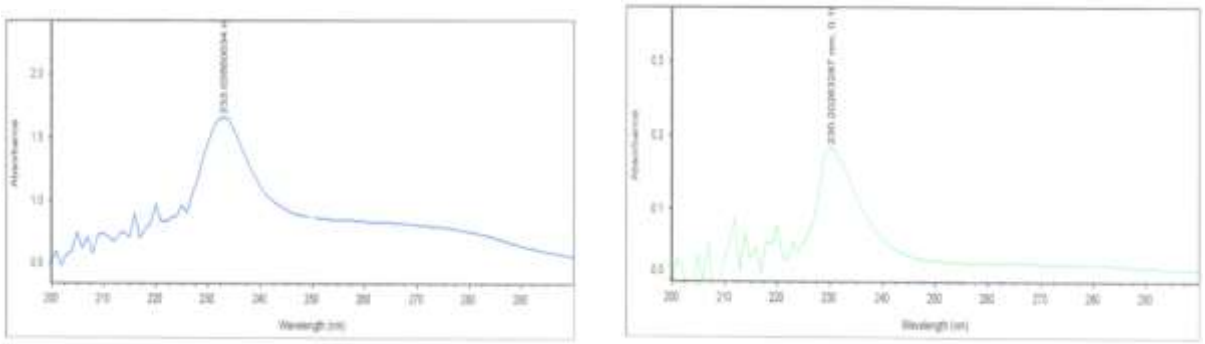


(a) (b)

Figure 1 SEM images of (a) crude collagen and (b) purified collagen

UV-visible Spectra of Extracted Collagen

Figure 2 (a) and 2 (b) show the UV-visible spectra of crude and purified collagen. The ultraviolet spectra of extracted crude and purified collagen showed the maximum absorbance near 232 nm. Moreover, a shoulder or smaller peak in the 250-290 nm region is absent because of the negligible amount of tyrosine residues and the absence of tryptophan in the extracted collagen.



(a) (b)

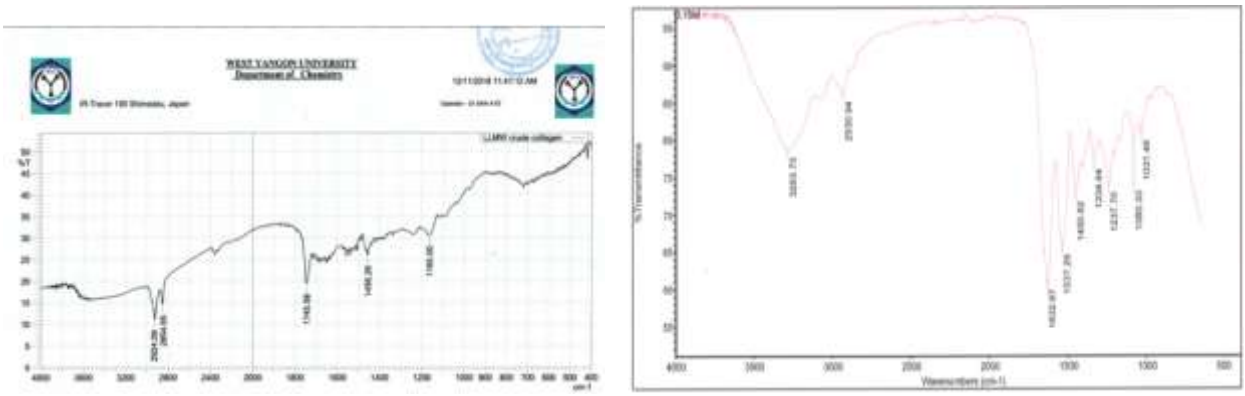
Figure 2 UV-visible spectrum of (a) crude collagen and (b) purified collagen

Fourier Transform- Infrared (FT IR) Analysis

The FT IR spectroscopy provides information regarding interaction via analysis of FT IR spectra corresponding to stretching or bending vibrations of particular bonds. Figure (3, a) shows the FT IR spectra of extracted crude collagen. The amide B band at 2924 cm^{-1} was assigned to the asymmetrical CH_2 stretching. The amide II band, 1456 cm^{-1} was assigned to the NH bending coupled with CN stretching vibration. The peak of amide III band was found at 1165 cm^{-1}.

Figure (3, b) shows FT IR spectra of purified collagen. According to Doyle (1975), a free N-H stretching vibration occurs in the range of 3400-3440cm^{-1} and when the N-H group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies. The amide A

band of purified collagen was found at the 3283 cm^{-1} was assigned to the hydrogen bonding in the NH group of the peptide, which is the main functional group of the collagen. The amide B peak at 2930 cm^{-1}, was represented the asymmetrical stretching of CH_2. The amide I band was found at 1632 cm^{-1}, which associated with the stretching vibration of carbonyl groups (C=O) along the poly peptide backbone. The amide II at 1537 cm^{-1} was assigned to the NH bending coupled with CN stretching vibration. The amide II band indicates the secondary structure of collagen. The amide III band, 1237 cm^{-1} was related to triple helical structure of collagen.

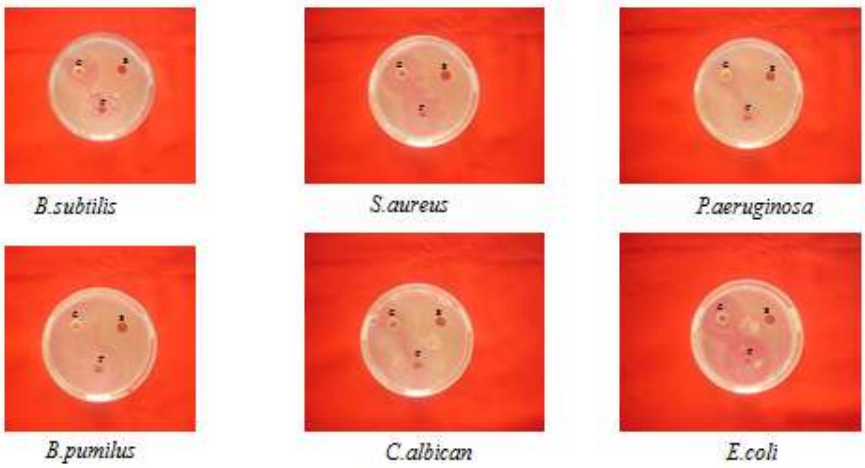


(a) (b)

Figure 3 FT IR spectrum of (a) crude collagen and (b) purified collagen

Antimicrobial Activity of Collagen

Antimicrobial activity of crude collagen and purified collagen was investigated against six species of microorganisms by employing agar well diffusion method. The samples were tested on six species of microorganisms including *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans and Escherichia coli*. The results are shown in Figure 4 and Table1. It was found that crude and purified collagen showed highest antimicrobial activity against all tested microorganisms with inhibition zone diameter higher than 20 mm. So, it may be inferred that the collagens extracted from the chicken feet can be effective in the formulation of medicine for the treatment of diseases such as soft tissue infection, bone and joint infection, ear infection, burn infection and as a surgical homeostatic agent.



C= Crude collagen, P = Purified collagen, S = Solvent (Acetic acid)

Figure 4 Antimicrobial activities of crude collagen and purified collagen against six microorganisms

Table 1 Antimicrobial Activity of Crude and Purified Collagen

	Inhibition zone diameter (mm)					
Samples	***B.subtilis***	***S.aureus***	***P.aeuginosa***	***B.pumilus***	***C.albican***	***E.coli***
Crude collagen	36 (+++)	35 (+++)	40 (+++)	35 (+++)	38 (+++)	35 (+++)
Purified collagen	38 (+++)	37 (+++)	40 (+++)	36 (+++)	40 (+++)	36 (+++)
Control (10 % acetic acid)	-	-	-	-	-	-

Agar well -10 mm
10 mm ~ 14 mm (+) → normal
15 mm ~ 19 mm (++) → high
20 mm above (+++) → highest

Biomedical Application of Chicken Feet

This test was carried out at Department of Medical Research (DMR). The incision skins of rats were treated with sofre tulle, crude collagen, purified collagen and commercial collagen.The progress of the treatment was recorded at a specified time interval. The results were shown in Figure 6. The healing rate of burned and scabs drop time were recorded on days 0, 2, 9 and 17. On days 2, the wound area increased initially and the progress of all burned skins were not significantly observed. On days 9,crude collagen, purified collagen and commercial collagen treated wounds releaved conspicuous redness on the wound border which is a sign of vascularization and re-epithelization. On days 17, shaving was performed on all rats, since hair had grown extensively. The burn wounds were almost completely healed that were treated with crude, purified and commercial collagen. From the observation crude and purified collagens have been shown to significantly accelerate the burn wound healing. After 17 days, histopathological finding of the skin lesions was carried out by haematoxylin and eosin (H & E) method and recorded by using light microscope with specific images. The results showed that the treatment with purified collagen and commercial collagen were better than the crude collagen and sofre tulle. This histopathological section of rat skin showed incomplete epithelialization in epidermis, dermis and subcutaneous tissue in no treatment group. Standard group of rat skin showed re-epithelialization of healing process but focal epidermal lesion is still present. The wound healing is still in progress and partially developed re-epithelialization of healing process and focal epidermal lesion is still present in epidermal layer by treatment with crude collagen and commercial collagen. Good wound healing in skin lesion of rat model was observed because presence of well-developed granulation tissues accompanied with well-developed sebaceous glands and sweat gland and hair follicles in epidermis and dermis layers of skin by the treatment with purified collagen. Better degree of wound healing was observed in this group by treatment with purified collagen than that of other. These results were showed in Figure 7.

Figure 5 Preparation of burn wound on rat model

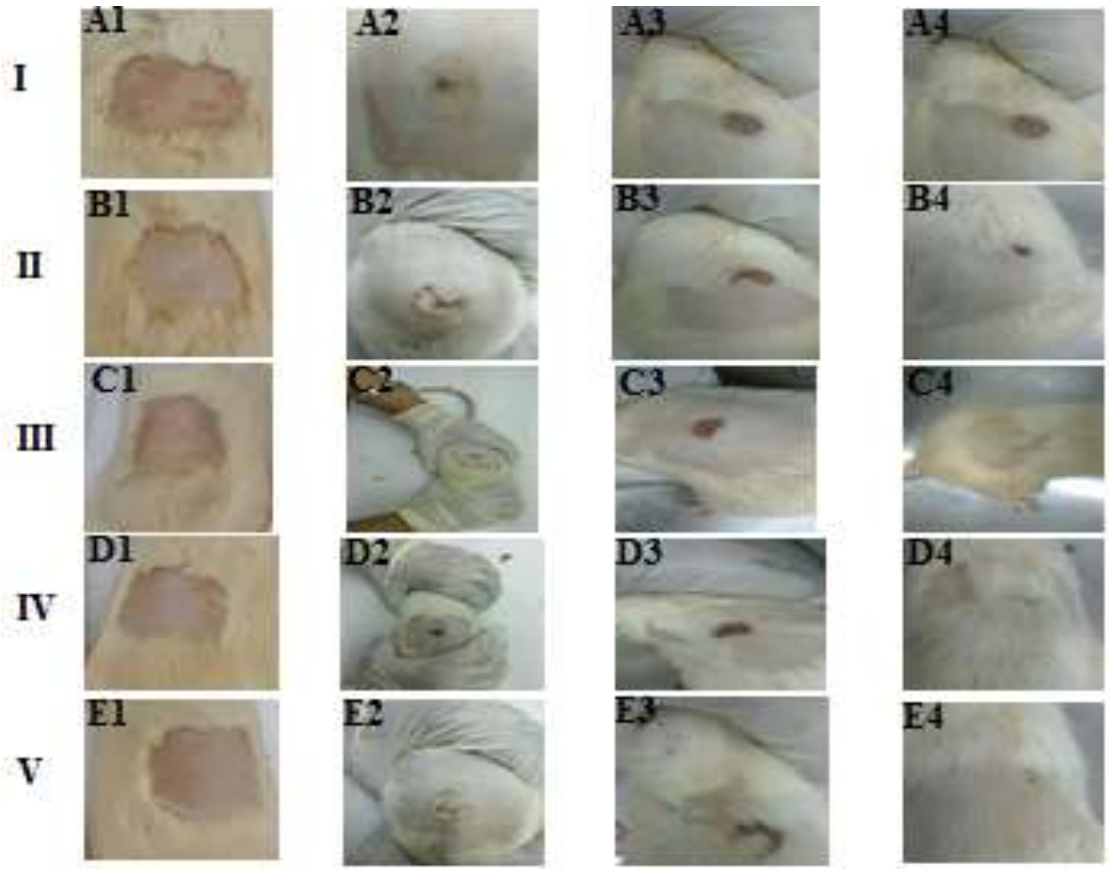


Figure 6 Burn wound activity of treatment on 17 days (I control, II sofre tulle, III crude collagen, IV purified collagen and V commercial collagen)

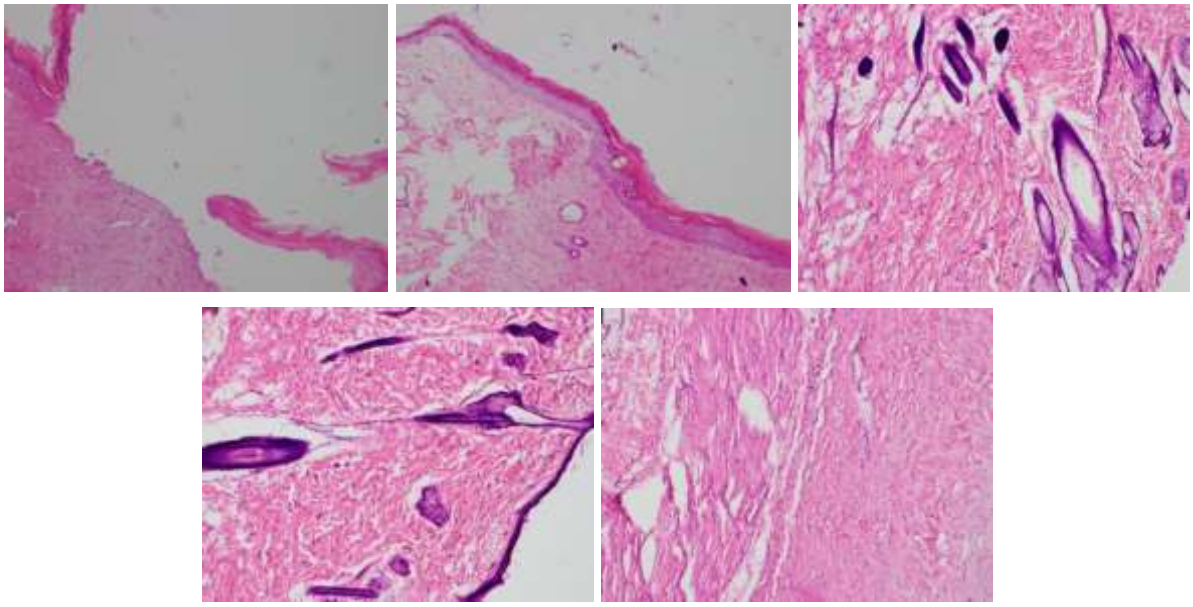


Figure 7 Hematoxyline and eosin-stained section of biopsies for the morphological evaluation of skin lesions of burn skin treated with crude collagen, purified collagen and commercial collagen on days 17

Conclusion

In this research, chicken feet were used as a source of collagen for biomedical application. Collagen was extracted from chicken feet by method of Liu, 2001 with slight modification and then crude collagen was purified by the method of Lin, 2013. The yield percent of crude and purified collagens were found to be 10.08 and 8.23 %, respectively. The micro architecture of collagen was studied by using Scanning Electron Microscopy, SEM. From the SEM result, both crude and purified collagens were identified as fibrillar structure. According to UV spectrum analysis, collagens were found to be the highest intensity of absorbance peak is 232 nm and no more other peaks were observed. The FT IR spectra data of collagen indicated the presence of N-H, C=O, C-H, O-H, CH_2. From the screening of antimicrobial activity of collagens, it can be concluded that the collagens showed the pronounced antimicrobial activity against all tested microorganisms. So, it may be inferred that chicken feet collagen can be used in the biomedical and pharmaceutical fields as a potential material for construction of tissue engineering scaffold and wound dressing system. The chicken feet collagens were studied in biomedical field especially burn wound healing compared standard sofre tulle drug. The significant improved in burn contraction by visually was observed when using the chicken feet collagen on days 17 after burning. Histopathological finding under microscope also reported that the skin treated with collagen exhibited good wound healing and well developing granulation tissue composed of sebaceous glands, sweat glands and follicles in epidermis and dermis layers. From these results chicken feet collagen was better than the standard sofre tulle drug for burn wound healing. Therefore, this research contributes to academic as well as biomedical application.

Acknowledgements

The authors would like to express their profound gratitude to the Department of Higher Education (Lower Myanmar), Ministry of Education, Yangon, Myanmar, for provision of opportunity to do this research and Myanmar Academy of Arts and Science for following to present this paper.

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