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Collagen derived from a giant African snail (*Achatina achatina*) for biomedical applications

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Abstract: *Achatina achatina* (AA) is a rich source of collagen due to its large size, but it is underutilized. Type I collagen was extracted from AA to serve as an alternative to existing collagen sources. The collagen was extracted at varying alkaline and temperature conditions to determine the optimal parameters that would give a high yield of acid-soluble collagen. The extracted collagen was characterised using X-ray diffraction, Fourier transform infrared (FTIR) spectrometry, thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC) to confirm the integrity and purity of the extracted collagen. The type of collagen was determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The α -1, α -2, and dimer electrophoresis bands confirmed that the collagen is type I, and the XRD data supported the findings. The highest collagen yield was obtained at 4 °C for 48 h, which decreased with increasing temperature due to the instability of the protein in acid at high temperatures. A cytotoxicity test was conducted using an Alamar blue assay. The AA collagen-treated normal prostate cell line (PNT2) showed no significant difference from the untreated control cells. The high-quality type I collagen extracted from AA has the potential for biomedical and other industrial applications.

Keywords: *Achatina achatina*; Type I collagen; characterisation

1. Introduction

Collagen is a triple helix of three polypeptide chains of molecules, mainly glycine, proline, and hydroxyproline. It is found in the extracellular matrix (ECM) of connective tissues, including tendons, ligaments, bone, and skin, in humans and other organisms [1]. Collagen in the ECM maintains structural integrity and also helps in the transmission of cellular signals [2,3]. Collagens are mainly produced within connective tissue by fibroblasts [4]. The literature reports 28 different types of collagens, which are grouped according to their function, composition, and location in the body.

Collagen is extracted from bovine and swine for biomedical applications for a variety of reasons, including aging and accidents [5]. For example, in the area of tissue engineering and regeneration, collagen is used in the design of artificial ECM

(scaffolds) to generate new tissues for the replacement of diseased or damaged tissues and/or organs in the human body [6]. In cosmetics, collagen is used to develop skin care products [7], among others. However, most of the existing and known sources of collagen extraction are notably expensive [5,8]. Literature reports on other low-cost sources of extracting collagen. These are poultry, fish, marine snails, and other marine animals. The yield of collagen extracted from these organisms is considerably small [9]. Moreover, the pre-treatment parameters and extraction conditions of collagen vary from one organism to another, depending on the organism from which it is extracted [10].

Non-collagenous proteins are removed from organic raw materials by breaking cross-links to allow acid to penetrate and extract collagen molecules. The physical swelling of the samples indicates adequate alkaline absorption, allowing the cleavage of crosslinks between other proteins in the samples [11–13]. In the extraction of collagen, the type and concentration of reagent used are also varied depending on the organism from which the collagen is being extracted [10]. The pre-treatment time also varies from a few hours up to two days [14,15]. Such noted variation in the temperature conditions is due to the temperature of the habitat of the organism from which the collagen is being extracted [14]. The extraction temperature conditions and the respective durations for which various extractions are carried out also vary. The most preferred temperature for extracting collagen is 4 °C. However, there are also reports of collagen extraction carried out at 8 °C, 25 °C, 37 °C, and 50 °C [16].

Literature reports of the extraction of gelatin from marine snails (*Hexaplex trunculus*), which are molluscs and bear some level of structural similarity to giant African snails (*Achatina achatina*). Therefore, we extracted collagen from an onshore source, *Achatina achatina*, to validate the pre-treatment and extraction conditions. The giant African snail is rich in protein [17] and mostly regarded as a pest [18,19] in some parts of sub-Saharan Africa while others consume its meat as a delicacy [17]. This research seeks to use the giant African snail as a replenishable and more affordable source of collagen due to its abundance and comparatively high reproductive rate [20,21].

The snail foot muscle (meat) was removed from the snail shell, washed, minced, and pre-treated with alkaline at different molar concentrations for different time intervals, after which the collagen extraction was achieved at different temperatures. The extracted collagen was characterised using X-ray diffractometry (XRD), Fourier transform infrared (FTIR) spectrophotometry, thermal gravimetric analysis (TGA), and differential scanning calorimetry (DSC) to determine the phase purity, functional groups, and thermal stability of the extracted collagen, respectively. Also, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the type of collagen present, after which a cytotoxicity test was performed.

2. Materials and methods

2.1. Materials

The materials included acetic acid (99% purity), sodium hydroxide (NaOH) and butanol (C₄H₉OH) (Philip Harris Education, Cheshire, England), hydrochloric acid (HCl) (WWR International Ltd., Fontenay-sous-Bois, France), sodium chloride

(NaCl), trizma-hydrochloric (trizma HCl) acid, proteases, glycerol, sodium dodecyl sulphate (SDS), Coomassie blue (Merck KGaA, Darmstadt, Germany), tris acetate EDTA (TAE) and tris borate EDTA (TBE) (Central Drug House, P, Ltd., New Delhi, India), and lysis buffer (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

2.2. Pre-treatment and extraction

Achatina achatina (AA) snail was obtained from the Ashaiman local market in Ghana. The shells were removed, and the meat was washed with chilled, double-distilled water. The foot of the snail meat was shredded into 0.5×0.5 cm² pieces using a scalpel blade and stored at 4 °C until it was ready for use. A mass of 30 mg each of the minced meat (MM) was added to four different concentrations of NaOH (0.05, 0.1, and 0.2 M, respectively) at a ratio of 1:20 (w/v). The mixture was stirred at 4 °C using a Stuart Magnetic Heat Stirrer-UC152 (Cole-Palmer, Staffordshire, United Kingdom) for 6, 12, 24, and 48 h. The mixture was maintained at 4 °C in a 1500-mL beaker. The ice was crushed into the beaker using an Ice Crusher IC20 (Waring Commercial, USA) to create an ice bath. The NaOH was replaced every 3 h, and the MM was continuously observed for sufficient physical swelling. The MM was sieved with a double cheesecloth and rinsed thoroughly with chilled (4 °C) double distilled water to remove NaOH and obtain a pH of 7.3. The pH was monitored with a Mettler Toledo pH metre (N.V. Mettler-Toledo, Zaventem, Belgium), and the respective level of observable swelling of the MM within the given duration was noted.

Fats and pigments were removed from the pre-treated minced foot muscle (PMM) using 10% butanol at 4 °C under continuous stirring in the ratio 1:20 (w/v) [16,22]. The butanol was replaced every 2 h for 6 h. A double cheesecloth was used to sieve the PMM, which was then thoroughly rinsed with chilled double-distilled water to remove the butanol and obtain a defatted and decoloured minced foot muscle (DMM) of the PMM. The DMM was weighed with a Denver Instrument electronic balance (Cole-Palmer, Court Vernon Hills, IL, USA) to obtain the wet weight.

The extraction of collagen from DMM was carried out using 0.5 M acetic acid at a ratio of 1:20% (w/v). Four sets of extraction were carried out at varying temperatures (4, 8, 16, and 32 °C) for four different durations (12, 24, 36, and 48 h), respectively, in 250-ml beakers [23,24]. For 4 °C collagen extraction, 20 g of the DMM was used for 12, 24, 36, and 48 h. An ice bath was created using a 1500-mL beaker, and a 250-mL beaker containing the DMM and 0.5 M acetic acid was placed into it. For 8 and 16 °C, the temperature was controlled in the ice bath. For 32 °C, DMM in the beaker was placed on a Stuart Magnetic Heat Stirrer (UC152) (Cole-Palmer, Staffordshire, United Kingdom) and stirred for all durations (12, 24, 36, and 48 h). The DMM was sieved using double cheesecloth, and the filtrate was collected after the respective durations. The filtrate in each case was precipitated with 2.5 M NaCl in the presence of trizma-HCl and centrifuged at 10,000 rpm for 10 min. The residue was collected in the form of collagen concentrate (CC). The CC was dialyzed against 0.1 M acetic acid using a cellophane dialysis tube for 24 h and dialyzed again against double-distilled water for 36 h. The dialysis was carried out at 4 °C [25,26] and the dialyzed CC was lyophilized to obtain collagen powder using a vacuum freeze dryer (Laconco

Corporation, Kansas City, U.S.A.). The collagen powder (CP) in each case was weighed to obtain the dry mass. The wet yield of the collagen in Equation (1) was obtained by dividing the weight of the collagen obtained after freeze-drying by the wet weight and multiplying the result by 100.

$$Yield = \frac{(\text{weight of collagen obtained})}{(\text{wet weight})} \times 100 \quad (1)$$

2.3. Characterization techniques

X-ray diffractometry (XRD, PANalytical Empyrean, Netherlands) was used to determine the phase purity of the extracted collagen. The XRD pattern of the collagen was obtained using CuK α radiation of wavelength, $\lambda = 1.5406 \text{ \AA}$. CP were scanned from 5° to 100° with a scan step size of 0.05° . Fourier transform infrared spectrometry (FTIR) data was recorded with a Nicolet MAGNA-IR 750 spectrometer (Nicolet Instrument Co., Madison, WI, USA). The analysis was done from 450 to 4000 cm^{-1} and the data was analysed. The CP thermal profile was analysed using a thermogravimetric analyzer and differential scanning calorimetry (TGA-DSC, Q600 SDT, TA instrument). The thermal profiles were obtained at a temperature range of 0 to $700 \text{ }^\circ\text{C}$ at a heating rate of $10 \text{ }^\circ\text{C}/\text{min}$ under nitrogen gas at a flow rate of $100 \text{ cm}^3/\text{min}$. The type of the CP was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the Laemmli method was used with a little modification [27]. This modification entailed mixing the extracted collagen with Tris-HCl buffer containing 2% mercaptoethanol and 0.016% bromophenol blue to reach a final collagen concentration of 1 mg/mL and boiling the mixture for 5 min at $95 \text{ }^\circ\text{C}$. Each boiled EC ($15 \text{ }\mu\text{L}$) was injected into the stacking gel and electrophoresed under a current of 12 mA. The gel was stained for 45 min in the presence of a 0.25% Coomassie Brilliant Blue R-250 solution and destined for a 5% methanol/7.5% acetic acid solution until the bands were clear. Two gel densities, 16% and 12%, were used for the electrophoresis. The device used was a BIO-RAD mini-PROTEAN Tetra System (Bio-Rad Laboratories, Dubai, U.E.A.) [28,29].

2.4. Cell viability assay (Alamar Blue assay)

The effect of the extracted collagen on cell viability and proliferation was determined using an Alamar Blue assay. The normal prostate cell line PNT2 (Sigma Aldrich) was seeded at 10^4 cells/well into a 96-well plate and incubated for 24 h. AA collagen was then administered to the cells in varying concentrations for 24, 48, and 72 h. After time elapsed, Alamar Blue[®] reagent was added to each well, including the negative controls and blanks, and incubated for 4 h at $37 \text{ }^\circ\text{C}$ away from light. Using the VarioskanTM LUX multimode microplate reader, fluorescence at an excitation wavelength of 560 nm and an emission wavelength of 590 nm was obtained. The cell viability was calculated using the formula

$$\% \text{ Cell viability} = \frac{\text{Relative fluorescence units of tested sample}}{\text{Relative fluorescence units of untreated cell control}} \times 100 \quad (2)$$

2.5. Statistical analysis

A two-way ANOVA Sidak's multiple comparisons was used to determine the

statistical significance, which was determined at a confidence level of $p < 0.05$. All the data presented were averages (mean) with their standard deviations as errors.

3. Results

3.1. Pre-treatment

A 0.05 M NaOH lower concentration used led to low swelling of the MM. However, the MM treated with 0.1 M NaOH for 6 h swelled moderately, and for 12 h, the swelling was highly significant. From 12 to 24 h, the physical structure of the MM started to overs well and become gel-like. A 0.2 M NaOH concentration led to the swelling of MM early on in the experiment, but the sample started to physically dissolve gradually into the solution, thereby making the solution cloudier from around 12 to 24 h.

3.2. Extraction of collagen

The highest quantity of collagen weighed was obtained at 4 °C for 48 h followed by 8 °C for 48 h, and the least quantity obtained was at 32 °C for 12 h. At 36 and 48 h, no significant quantity of collagen was obtained at 32 °C, as most of the MM started to gelatinize in the acetic acid, turning it into jelly. Collagen extracted at 8 and 16 h for various durations resulted in comparatively lower yields. **Figure 1** shows the average yield of collagen extracted at the respective temperatures.

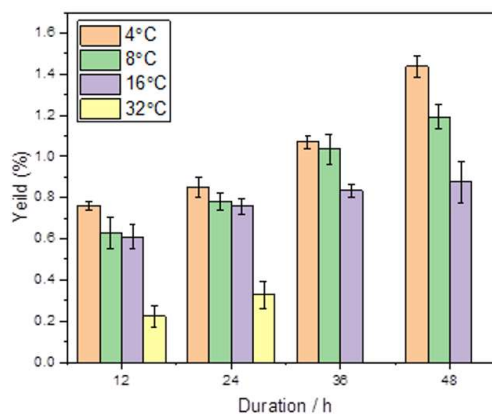


Figure 1. Graphical representations of the collagen yield extracted at 4, 8, 16, and 32 °C.

3.3. XRD results

Figure 2 shows the XRD pattern of CP extracted from *Achatina achatina*, AA. The patterns possess three main peaks occurring at $2\theta/\text{degrees}$ (8.3° , 21.8° , and 32.1°) and correspond to XRD patterns of standard collagen [30]. The first peak that occurs at 8.3° is the highest, and it indicates the distance between the molecular chains with the triple helical amino acid. This peak is, however, not visible in the pattern of collagen extracted at 32°C . The peak of the CP, occurring at 21.8° , is broader than the other two peaks and corresponds to the amorphous scattering that resulted from disorganised components of the collagen fibres. The smallest peak on the pattern

occurring at 32.1° is due to the typical triple-helix structure occurring in collagen [31]. A similar peak is also seen in the XRD spectrum of collagen extracted from fish [32].

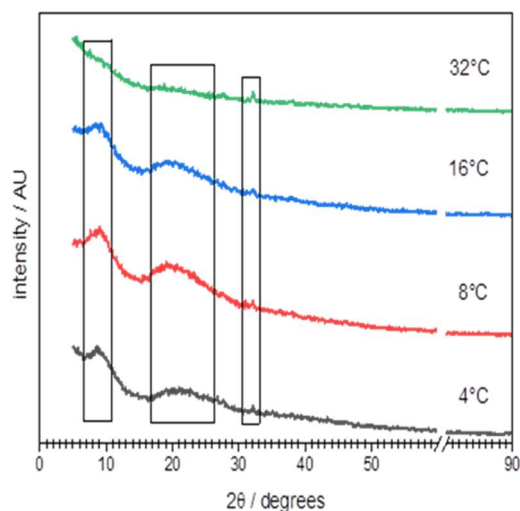


Figure 2. XRD spectrum of collagen extracted from *Achatina achatina*.

3.4. FTIR results

Figure 3 shows the FTIR spectrum of CP extracted from AA at 4, 8, 16, and 32 °C. The spectra show bands identical to those of the golden apple snail (*Pomacea canaliculata*) [33]. The CP extracted from the four main temperatures showed characteristic patterns in regions of amide A, amide I, amide II, and amide III. The amide A bands occurring around $3600\text{--}2300\text{ cm}^{-1}$ occur as a result of N–H stretching, while the amide I bands, which are formed at $1656\text{--}1644\text{ cm}^{-1}$ are due to C=O stretching. The bands at $1560\text{--}1335\text{ cm}^{-1}$ are amide II, occurring because of N–H bending and C–N stretching vibrations. The amide III bands at $1240\text{--}670\text{ cm}^{-1}$ resulted from an intricate system associated with CH_2 residual groups from glycine and proline [34].

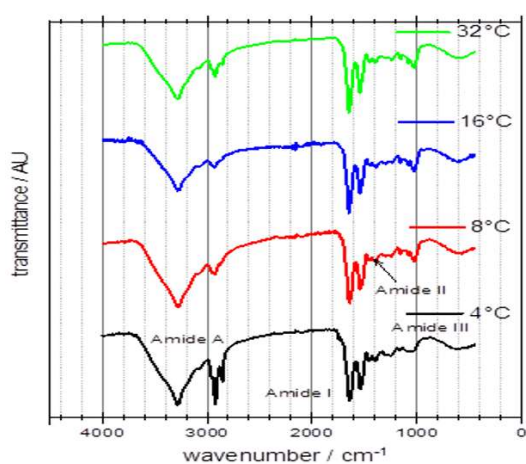


Figure 3. FTIR spectrum of collagen extracted from *Achatina achatina* at various temperatures.

3.5. TGA-DSC

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were used to determine the thermal stability of the extracted collagen. **Figure 4** depicts a DSC plot of heat flow (blue) versus temperature, with the CP beginning to receive heat (endothermic reaction) at 31.3 and rising to 229.5 to achieve a stable heat flow up to 700. The derivative heat flow (red) shows a corresponding chemical component that was given out from the heat reaction. At 51.25, the collagen started denaturing and losing structure water at 112.1.

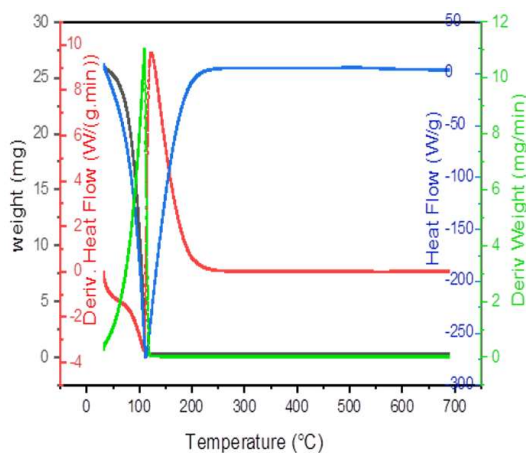


Figure 4. Shows the TGA-DSC profile of the derived collagen.

3.6. SDS-PAGE

CP with a gel [density of 12%] had its α -1 and α -2 chains occurring at 110 and 80 kDa, respectively, while the β -dimer occurred at 200 kDa. Other peptides also occurred in the CP in strong and weak bands. These bands occurred around molecular weights of 160, 44, 43, and 25 kDa, as shown in **Figure 5**. According to the literature [35], Sheep Trail Tendon, a type I collagen, has three signature bands on SDS-PAGE: the α -1 chain, the α -2 chain, and the β dimer. The bands are present in the CP extracted from AA [36,37]. The intensity of the α -1 chain band of the collagen extracted appears almost twice as heavy as what appears in the α -2 chain. The chains occur at \sim 115 and \sim 110 kD for the α -1 chains, and \sim 129 and \sim 125 kDa for the α -2 chains.

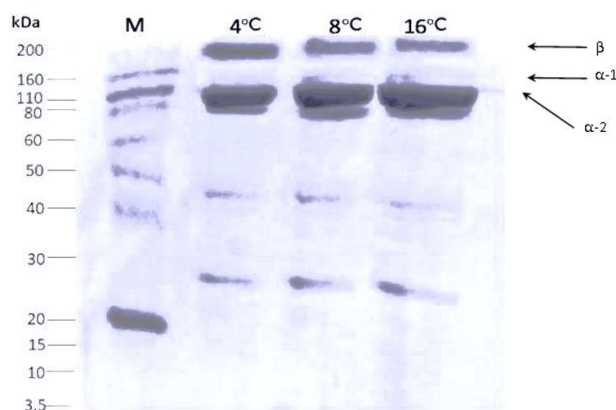


Figure 5. SDS-PAGE pattern of collagen extracted from *Achatina achatina*.

3.7. Cytotoxicity

The effect of AA collagen on the PNT2 cell line viability was determined using an Alamar Blue assay for 24, 48, and 72 h and showed no cytotoxicity in vitro. The plot in **Figure 6** shows various extract variations in Alamar blue with no significant toxic effect on PNT2. On high extract variation, a slightly significant difference was observed at 24 and 72 h.

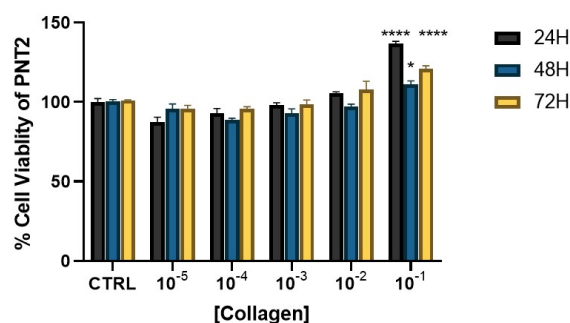


Figure 6. The effects of *Achatina achatina* foot muscle collagen on the normal prostate cell line PNT2 viability using the Alamar blue assay.

There was no significant effect (no *) of AA collagen at most concentrations on the cell line. A significant proliferation was observed only at the highest concentration. * = significant, **** = highly significant, CTRL = untreated control.

4. Discussion

Achatina achatina has been identified through studies to serve as both a host and an immediate vector for some parasitic species [38]. The link between *Achatina achatina* and parasites occurs through feeding and direct contact with the source of these parasites [39]. Works done [40] in different local government areas in Nigeria on the parasitic profile of five species of terrestrial *Achatina* snails indicated that parasitic prevalences with different species were different for the respective localities from which the snail samples were obtained, and the prevalence of parasites of *Achatina achatina* is dependent on the proximity of the parasite source. *Achatina achatina* has a comparatively high reproduction rate and is often reared in sub-Saharan Africa for consumption as food. *Achatina achatina* reared domestically will be less susceptible to parasite infestation than those captured from the wild.

NaOH was preferred for MM pre-treatment because HCl pre-treatment at 0.1 M results in an extremely low collagen yield. A higher concentration also produced a physical breakdown of the MM structure in the reagent. The pre-treatment was carried out to remove non-collagenous proteins from the MM, making it easy for the extraction of collagen [13,16]. Three factors that mainly influenced the pre-treatment of the MM were the concentration of NaOH, the duration for the MM to swell, and the temperature at which the swelling occurs. However, excessive swelling of the MM leads to the cleaving of cross-link chains in the non-collagenous proteins and peptide bonds in the collagen. The cleaving is temperature-, concentration-, and time-dependent. Comparatively long exposure to NaOH concentration at 4 °C led to the dissolution of MM under preferential conditions of 0.1 M NaOH for a duration of 12 h. The poor swelling observed in the MM pre-treated in 0.05 M NaOH is a result of the low concentration of the reagent used since a low temperature, less concentrated

NaOH, and relatively short duration of treatment also lead to poorly cleaved cross-links, poor swelling of the MM, and subsequently a low collagen yield [13,16]. A concentration that is higher than 0.05 M and a suitable duration and temperature would also aid in the considerable swelling of the AA MM. The 0.1 M NaOH pre-treatment produced a significant swelling within the first 12 h of continuous stirring at 4 °C. Other concentrations of the alkaline used to pre-treat the MM were therefore not appropriate because they either led to over-swelling or insufficient swelling. The appropriate swelling directly translates into collagen yield. The pre-treatment process used in this study contrasts with the pre-treatment process for the *Hexaplex trunculus* [36]. In their study, the sample was soaked in 0.02 M NaOH for 60 min at room temperature. In a study conducted [41], the collagen source was from the skin of the red snapper and was pre-treated with 0.1 NaOH for 12 h. Here, the study reported, after carrying out a quantitative analysis with Bradford and bovine serum albumin (BSA) tests, that the concentration of non-collagen protein was impacted. NaOH was also used by Blanco et al. [42] for the pre-treatment of their sample, and further multivariate analysis of their sample showed that temperatures around 4 °C and an alkaline concentration of 0.1 M led to a high yield of collagen during extraction. In their study, Blanco et al. [42], however, gave a range of 0.05 M to 0.1 M and temperatures from 4 to 20 °C for alkaline pre-treatment, depending on the source of collagen being extracted. The fat content in snail muscle is generally low [43]. Therefore, the procedure was geared more towards the removal of pigments, as Xu et al. [22] used H₂O₂ to decolor their sample, using a sample-to-solution ratio of 1:20 at 4 °C for 24 h, and suggested that the decoloring and degreasing process enhanced the purity level of the collagen extracted. Studies carried out by Kaewdang et al. [37] also used butanol to remove fats from yellowfin tuna samples before proceeding with the extraction process.

The extraction at 4 °C had the highest yield of around 1.44%, and the acid concentration and duration of the extraction were consistent with what was reported by Kaewdang et al. [37]. They also determined the yield of acid-soluble collagen extracted using the dry weight of yellowfin tuna to be 1.07%. The extraction of collagen from *Sheep tail tendon* carried out acid extraction and reported a yield of 9.7% which was obtained from 1.651 g of the dry weight of the tendon [44], while acid soluble collagen extracted from the mesoglea of jellyfish gave 0.12% of the wet weight [45]. A study that extracted acid-soluble collagen from Indonesian local goat skin estimated a yield of 7.35%, which was obtained after 48 h of treatment with acid [46]. The yield of collagen extracted from three different types of fish, namely common carp, red snapper, and milkfish was estimated to be between 0.8%–2.1% [45]. The differences in yields can be attributed to the different collagen structures in different organisms, the varying crosslinking of collagen fibrils in different by-products used, and the procedure of extracting the collagen [13].

A study conducted by Matmaroh et al. [47] reported the wet weight of collagen extracted from the cartilage of brown-banded bamboo sharks to be 1.27% [37].

Collagen extracted from various sources using the acid extraction method provides a fair idea with regards to the amount (yield) and abundant information about the nature (characteristics) of collagen that is present in a particular source. However, this method is not employed as a quantitative extraction method for collagen because

the literature provides evidence that the entirety of collagen present in a particular source cannot be extracted using only the acid extraction method. Other methods need to be used, or employed to complement this method, to obtain full quantitative information about collagen from a given source [27,48]. Acid extraction gives a low yield, while other extraction methods give higher yields. Examples of extraction methods that provide full quantitative information about collagen extracted from various sources include enzymatic and ultrasonic extraction methods.

The phase composition, functional groups, and thermal stability data from the XRD, FTIR, and TGA-DSC revealed that the extracted CP from the AA is collagen, specifically type I collagen, as indicated by the results from the SDS-PAGE. The most common types of collagens in the human body are types I, II, III, IV, and V. Type I collagen is the most abundant type (about 90%) in the human body and is found in the scar tissue, bone, tendons, ligaments, and the skin in the arteries to support smooth, firm, and strong body tissues [49,50]. The results show that the extracted collagen has no significant toxic effect on the PNT2 cell line. Most cell lines are cultured in collagen-coated vessels, and they have been found to be a suitable modification for culture vessels [51]. Compared to the untreated control, collagen increased proliferation of the cell line at the highest concentration, which corresponds to results found in the literature [52].

5. Conclusion

Collagen was extracted from the foot muscle of the giant African snail (*Achatina achatina*) using the acid-soluble extraction method. The giant African snail serves as a viable source of collagen for biomedical and other applications. The optimum pretreatment was a 0.1 M NaOH solution for 12 h at 4 °C under constant stirring. The other pretreatment conditions lead to the cleaving of cross-links, which swells the sample too fast and thus damages it. The extraction was done using 0.5 M acetic acid at 4 °C for 48 h, and the highest yield of the extraction was 1.44%. The phase purity, functional groups, and thermal stability of the extracted collagen were characterised by X-ray diffractometry (XRD), Fourier transforms infrared (FTIR) spectrophotometry, thermal gravimetric analysis (TGA), and differential scanning calorimetry (DSC), respectively, whereas sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) determined that the collagen was type I. The collagen had no significant effect on the normal prostate cell line, PNT2.

Author contributions: Conceptualization, BOA and DP; methodology, DP, SKK and BSK; software, BOA; validation, BOA, DP, SKK and BSK; formal analysis, BOA, DP and BSK; investigation, DP and BSK; resources, BOA, BM, VAA; data curation, BOA; writing—original draft preparation, BOA, DP, LP and BSK; writing—review and editing, BOA, EJF and EKT; visualization, BOA; supervision, BOA, LP and EKT; project administration, BOA; funding acquisition, BOA, DP and EKT. All authors have read and agreed to the published version of the manuscript.

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