A Study on the Synergistic Effect of Bioactive Compounds from Papaya Leaf and Collagen Isolated from Indian Mackerel on *in vitro* Wound Closure Capacity

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ABSTRACT

Collagen is an extracellular matrix protein which plays a vital role in the wound healing process. Wound healing properties of papaya leaf extract have been well studied. Therefore, the synergistic effect of papaya leaf phytoconstituents and isolated collagen was evaluated for its wound healing properties. In the present study, acid-soluble collagen (ASC) was isolated from the skin of Indian mackerel (*Rastrelliger kanagurta*) and characterized. Along with isolated ASC, major papaya leaf constituents including caffeic acid, coumaric acid, quercetin and oleic acid were added to study their effects on wound healing. The ASC was isolated with a yield of 3.57 %. On the basis of SDS-PAGE analysis, the isolated ASC was identified as type I collagen. In the FTIR spectra, five amide groups were identified as major bands, which represents the triple helical nature of the collagen. By SEM analysis, the collagen fibers were found to be densely packed. Different combinations of collagen gel and papaya leaf compounds were prepared by one-to-one combination as well as mixed combinations. These test compounds were screened by *in vitro* scratch wound assays using the NIH3T3 cell line. Among all the test compounds, collagen with quercetin (0.04 mg) was the most effective in enhancing wound closing, with 100 % migration of fibroblasts.

Keywords: Acid-soluble collagen, Indian mackerel, Papaya leaf, Wound closure

INTRODUCTION

Wound healing is an essential biological phenomenon that involves tissue repair and regeneration. *Carica papaya* belongs to the Caricaceae family, possesses antimicrobial, antioxidant and anti-inflammatory properties, and also exhibits excellent wound healing activity. Major phytochemicals such as alkaloids, flavonoids, glycosides, phenolics, and saponins, etc., have been isolated from *C. papaya* leaves. Important phenolic compounds found in papaya leaf extracts include caffeic acid, p-coumaric acid, quercetin, protocatechuic acid, 5, 7-dimethoxy coumarin, kaempferol and chlorogenic acid, which act as free radical terminators (Canini *et al*., 2007; Aruljothi *et al*., 2014). Enormous reports are available associated with the wound healing capabilities of leaf extracts. However, there are limited reports available on the synergistic effect of individual leaf constituents along with extracellular matrix (ECM) proteins such as collagen. Therefore, in the present study, specific leaf constituents along with collagen gel were evaluated for their wound healing activity.

Collagen plays a vital role in various phases of wound healing, such as hemostasis, proliferation, inflammation and remodeling, and it

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Received 12 July 2019 / Accepted 4 February 2020

attracts fibroblasts and keratinocytes to the wound (Weber *et al*., 1984; Brett, 2008). During the inflammatory phase of normal wound healing, proteases such as matrix metalloproteinases (MMPs) are recruited to the wound, where they remove inflamed ECM and replace it with new tissue forms (Parks, 1995). In contrast, elevated levels of MMPs for a prolonged period may cause damage to healthy ECM, which delays wound healing (Vowden, 2011). Therefore, alternate processes are needed to balance the excess MMPs during wound healing. This suggests the application of collagen-containing gels, pastes, polymers, films and hydrogels in the management of delayed wound healing (Rangaraj *et al*., 2011). Collagen dressings support the formation of new collagen strands in the wound, which stimulate new tissue development.

The collagen in dressings interacts with MMPs. MMPs normally attack and break down collagen, therefore collagen-containing wound dressings act as an alternative collagen source for MMPs, which leaves normal collagen available for wound healing (Brett, 2008). Another useful aspect of a collagen dressing is its ability to reduce the occurrence of wound infection. It can help by creating a barrier layer between the wound and the surrounding bacteria, as well as spreading antimicrobial agents around the injury, reducing the overall risk of infection.

Herein, acid-soluble collagen (ASC) was isolated from the skin of Indian mackerel (*Rastrelliger kanagurta*), which belongs to family Scombridae. It is commonly utilized in south and south-east Asian cuisines, and it is rich in protein, essential amino acids and the antioxidant coenzyme Q10. It has a variety of biomedical applications. Therefore, the collagen isolated from the skin of Indian mackerel is used as a source of collagen for diverse applications. Type I collagen is used as a biomaterial for the wound healing process rather than the other types of collagen as it is having high cell- adhesion properties followed by low antigenicity (Pati *et al*., 2010). The stability of isolated collagen was improved by chemically cross-linking with additives, which reduces the rate of enzymatic and hydrothermal degradation. The main objectives of the present study was to isolate collagen from the skin of Indian mackerel and to examine the wound closure effects of major papaya leaf constituents such as caffeic acid, coumaric acid, quercetin and oleic acid, along with collagen gel by *in vitro* scratch assay study using the NIH3T3 cell line.

MATERIALS AND METHODS

Materials

The chemicals used in the present study were of analytical grade. Acrylamide, ammonium per sulphate, bovine serum albumin (BSA), caffeic acid, coumaric acid, EDTA, Folin-Ciocalteu reagent (FC), oleic acid, sodium dodecyl sulphate, Tris (hydroxyl methyl) amino methane, and quercetin were procured from Merck®, India. High-molecular-weight markers in the range of 10 kDa to 245 kDa were procured from Sigma Chemical Co., St. Louis, Mo, USA. DMEM HG (Dulbecco's Modified Eagle Medium, High glucose), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), and DMSO (Dimethyl sulfoxide) were procured from Invitrogen. NIH3T3 cell lines were procured from the National Centre for Cell Science (NCCS), Pune, India.

Fish sample collection

Indian mackerel were procured from the fish market at Mangalore. The skin portions of the fish were removed and washed with cold distilled water and stored at -20 °C until further use.

Instrumental analysis

Structural confirmation of ASC was done by FTIR Spectrophotometer (IR Prestige- 21, Shimadzu Corporation, Japan). The spectra was measured within the frequency range of 4000-500 cm^{-1}. The surface morphology of ASC was analyzed by Field Emission Scanning Electron Micrograph (Carl Zeiss Microscopy Ltd). The lyophilized collagen sample was cut using a punch and fixed to an adhesive carbon stub. The sample was sputtered with gold particles under vacuum to

increase electrical conductivity and operated at 5 kV. Thermogravimetric analysis (TGA) was carried out to analyse thermal decomposition of isolated ASC using a thermal analyser (Model; SDTQ 600, TA Instruments, UK). The thermal scanning was carried out at a uniform scanning rate of 10 °C·min^{-1} over the temperature range of 25-700 °C. The UV-Visible absorption spectrum of isolated ASC was recorded by a spectrophotometer (Spectroquantpharo 300) at different wavelengths (200-800 nm).

Isolation of ASC from the skin of Indian mackerel

The acid-soluble collagen was extracted from the skin of Indian mackerel (*Rastrelliger kanagurta*) using the method reported by Nagai and Suzuki (2000a) with a little modification. All the preparation procedures were performed at 4 °C. The fish skins were removed for experimental samples, weighing a total of 50 gm. The samples were washed with cold distilled water for a few hours and ground. The ground sample was then extracted with 0.1 M NaOH (1:10 w/v) for 48 h to get rid of non-collagenous proteins. The process was repeated every five hours to ensure complete removal of non-collagenous matter. The residue was washed with cold distilled water until pH was close to neutral. Fat was removed with 10% butyl alcohol (1:10 w/v) followed by a wash with cold distilled water. The residue was extracted with 0.5 M acetic acid for a period of three days. The insoluble matter was re-extracted at a sample/acid ratio 1:2.5 (w/v) for two days with the same solution. Each extract was filtered by cotton cloth followed by centrifugation for 30 min at 10,000 rpm. The supernatant was mixed and salted out by adding NaCl to a final concentration of 2.5 M in the presence of 0.05 M Tris-HCl, pH 7.0, stirred well and kept overnight to precipitate the collagen. The resultant precipitate was collected by centrifuging at 10,000 rpm for 30 min. The pellet obtained was dissolved in 0.5 M acetic acid and dialysed against 0.1 M acetic acid and distilled water, respectively. Amicon Ultra centrifugal filters (100 kDa cut-off, Millipore) were used for concentrating the proteins. 10 mL of dialyzed collagen solution was taken in an Amicon centrifugal tube and centrifuged at 5,000 rpm for 15 min at 4 °C. Retentate was collected and then lyophilized. It was labeled as concentrated acid-soluble collagen (ASC) and stored at 4 °C.

Collagen yield (dry basis) was calculated by the following formula (Chen *et al*., 2016):

Collagen yield (%) = (Weight of final collagen sample in g)/ (weight of sample in g) × 100.

Protein concentration of ASC was determined by Lowry's method (Lowry *et al*., 1951), using BSA as a standard. Standard and samples were incubated with alkaline copper sulphate solution for 10 min at room temperature, followed by Folin-Ciocalteu (FC) reagent for 30 min. Absorbance was measured at 670 nm by spectrophotometer (Systronics-106). From the BSA standard graph, protein concentration of the samples was determined.

Effects of pH and NaCl on collagen solubility

The isolated collagen samples were solubilized in 0.5 M acetic acid (3 mg·mL^{-1}) and stirred at 4 °C until collagen was completely dissolved (Veeruraj *et al*., 2013). Collagen was dissolved in 0.5 M acetic acid and the pH was adjusted to final pH of 2, 4, 6, 8, 10 and 12; the volume was made up to 5 mL using distilled water.

To evaluate the effect of NaCl, collagen was mixed with cold NaCl (0%, 2%, 4%, 6%, 8%, 10% and 12% w/v). In both experiments, solutions were stirred for 30 min at 4 °C and centrifuged at 10,000 rpm at 4 °C for 30 min. The protein content from the supernatant was determined by Lowry's method (Lowry *et al*., 1951). The solubility of collagen was calculated in comparison with the different pH range.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of the ASC were analyzed by SDS-PAGE according to the method of Laemmli (1970), using 10% resolving gel and 6% stacking gel. Collagen samples were solubilized in 0.1 M

acetic acid together with sample buffer (0.125 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol, 1% Bromophenol blue and 5% (v/v) β-mercaptoethanol). The samples were heated at 90 °C for 5 min prior to loading the gel. The gel electrophoresis was induced at 50 V for 5 min, followed by 100 V for approximately 4 h. The protein bands were visualized with Coomassie blue R-250.

Gel formulations for in vitro wound healing activity using collagen with glycerol and papaya leaf constituents

In the present study, the synergistic effects of leaf constituents were evaluated along with extracted ASC. The tests were carried out as one-to-one combinations as well as mixed combinations. A series of gel samples were prepared with different constituents, as shown in Table 1.

MTT Assay on NIH3T3 cells

Cell viability in NIH3T3 cells was measured by a previously described, colorimetric assay, using MTT (Mosmann, 1983; Rajkumar, 2018). Briefly, 200 µL of the cell suspension was added to a sterile 96-well plate and incubated with 200 µL of test compounds for 24 h, then incubated with 100 µL of MTT reagent (10%) for 3 h. After incubation, 100 µL of DMSO was added. Absorbance was measured at 570 nm using a 96-well microplate reader. The percentage cell viability of test compounds compared to the control was calculated from the following formula:

Cell viability (%) = {(absorbance of test compound - absorbance of blank) / (absorbance of blank)} × 100.

A graph of percentage cell viability against concentration was plotted and IC_{50} was calculated (Muniandy *et al*., 2018).

Scratch assay

The NIH3T3 cells were trypsinized and centrifuged to obtain a cell pellet. A volume of 100 µL of cell suspension in DMEM was added to a 12-well plate and incubated at 37 °C with 5% CO_2 atmosphere for 24 h to reach ~100 % confluence as a monolayer. A scratch was gently made on the monolayer without changing the medium by using a micropipette tip across the centre of the well. The well was washed twice with DMEM to remove detached cells and replenished with fresh medium. The test compounds (25 µg) were added to the respective wells with 1 mL of fresh medium and the plate was incubated at 37 °C. Photos of scratched monolayers were taken under 40X magnification at zero and 24 h. Measurement of the scratch area at different time points was carried out using Image J 1.51j8 (Rajkumar *et al*., 2018). Percentage migration was calculated from the scratch area measurements using the following formula:

Percentage migration = {(Scratch area at 0 h − scratch area at 24 h) / (scratch area at 0 h)}×100.

Table 1. Gel formulations tested for wound closure activity.

Test compound	Composition of gel
Sample 1 (S1)	Collagen (5% w/v)+glycerol (2:1)
Sample 2 (S2)	Collagen+glycerol+0.25 mg caffeic acid
Sample 3 (S3)	Collagen+glycerol+0.34 mg coumaric acid
Sample 4 (S4)	Collagen+glycerol+0.04 mg quercetin
Sample 5 (S5)	Collagen+glycerol+0.25 mg caffeic acid+0.34 mg coumaric acid+0.04 mg quercetin
Sample 6 (S6)	Collagen+glycerol+0.25 mg caffeic acid+0.34 mg coumaric acid+0.04 mg quercetin+oleic acid

RESULTS AND DISCUSSION

Preparation of acid-soluble collagen

Collagen was isolated from the skin of *Rastrelliger kanagurta*. The isolated ASC sample was found to be odorless with fiber-like appearance. The yield (dry weight basis) of ASC was found to be 3.57 %. A comparative percentage yield of collagen from other fishes is listed in Table 2. Amounts of collagen varied by fish species, with differences mainly due to the degree of cross-linking among the collagen molecules (Foegeding *et al*., 1996). The obtained result was found to be lower than some other fishes. This may be due to the highly cross-linked structure of telopeptide region in the collagen, which makes the collagen less soluble in acid solutions. The differences in the collagen yield also depends on the type of fish species, age and physical properties such as temperature, extraction method, extraction time, influence of acetic acid and pH etc., (Duan *et al*., 2009; Ali *et al*., 2017). Even though the yield obtained for the isolation of ASC is comparatively less in the present work than for some other species, the product obtained seems to be qualitatively good, as it is evident from SEM images (Figure 5).

The protein content of ASC was found to be 34.68 $mg \cdot g^{-1}$. The total protein concentration is proportional to the color change of the sample solution by the addition of Lowry's reagent as a result of FC reagent reduction and oxidation of amino acids such as tryptophan, tyrosine, cysteine, histidine and asparagine (Lowry *et al*., 1951). As tryptophan is completely absent in collagen, proline is the major amino acid constituent that binds with Lowry's reagent and imparts color to the solution.

Solubility

Effects of pH and NaCl concentration on collagen solubility

The effects of the pH and NaCl concentration on the solubility of ASC are presented in Figure 1. The solubility of collagen was observed at pH 4 (highest solubility) and 10 (lowest solubility), respectively, as shown in Figure 1a, and similar to that reported in previous studies (Jongjareonrak *et al*., 2005; Liu *et al*., 2009). Collagen is soluble only at acidic pH, whereas at basic pH collagen gets precipitated. This is due to protonation of amines in the protein (Monsur *et al*., 2014); also, the region of increasing solubility coincides with the range of an increasing amount of protons present in collagen fibers.

Solubility in 0.5 M acetic acid increased gradually with increasing NaCl concentration, up to a level of 4%. A sharp decrease in solubility occurred when NaCl concentration was above 4%, as represented in Figure 1b. The solubility of collagen from the skin of trout, hake brownstripe, big-eye snapper and red snapper in an acetic acid solution decreased with increasing NaCl concentration (Jongjareonrak *et al*., 2005a; Kittiphattanabawon *et al*., 2005). Solubility of type I collagen from

Table 2. A comparative list indicating percentage yield of collagen from various fish sources.

Fish	ASC from skin (%)	Reference
Indian mackerel	3.57	Present work
Sepiella inermis	0.58	(Shanmugam *et al*., 2012)
Brama australis	1.5	(Sionkowska *et al*., 2015)
Black drum (*Pogonias cromis*) &	2.3	(Ogawa *et al*., 2003)
Sheepshead seabream	2.6	
Striped catfish	5.1	(Singh *et al*., 2011)
Seaweed pipe fish	5.5	(Khan *et al*., 2009)
Pufferfish (*Lagocephalus wheeleri*)	6	(Kirti and Khora, 2015)

snakehead scales decreased gradually with increasing NaCl concentration, up to 3%. The initial increase in the solubility with the increase in concentration of NaCl could be due to a "salting in" effect. Above this concentration, a sharp decrease in soluble protein concentration was observed due to a "salting out" effect (Liu *et al*., 2009; Tabarestani *et al*., 2012). High solubility of collagen opens a window for commercial applications. The results suggest that the extraction process yields collagen in the pure form.

Characterization

UV-Visible spectra

The absorption band of Indian mackerel skin collagen showed a major band at 225 nm and also a small band was found at 205 nm, as depicted in Figure 2. Similarly, previous studies revealed that the collagen isolated from carp exhibited a major absorption at 223 nm (Duan *et al*., 2009). UV absorbance spectra of collagen have been reported in the range between 220-230 nm (Na, 1986). Most of the protein absorbs UV light in the region around 280 nm and peptide bonds present in the protein absorb UV light below 210 nm (Stoscheck, 1990). The absorbance at 280 nm is mainly because of tyrosine, tryptophan and phenyl alanine residues, but in collagen, tryptophan is completely absent and it has negligible amounts of tyrosine. Therefore, absence of absorption at 280 nm indicated the purity of the isolated samples, and it also showed the effectiveness of alkaline treatment for removal of non-collagenous protein (Hema *et al*., 2013; Muralidharan *et al*., 2013). The observed result was similar to that of standard type I calf skin collagen, where an absorption band was found at 227 nm along with a small band at 205 nm.

FTIR analysis of ASC

FTIR spectroscopy is widely used to study the secondary structure of protein. It was carried out to investigate any differences in protein structure compared to previous reports. A FTIR graph is depicted in Figure 3, and shows five major peaks, namely amides A, B, I, II and III. Amides I, II and III are responsible for the triple helical nature of collagen. Amide A and B bands correspond to free -N-H stretching, which occurs in the range of 3,000-3,400 cm^{-1}. The amide I bands correspond to -C=O stretching vibration normally occurring in the range of 1,600-1,700 cm^{-1}. The amide II bands correspond to -NH bending and -CN stretching in the range of 1,550-1,600 cm^{-1}, while amide III bands correspond to -CN stretching and -NH in-plane bending vibration (deformation) in the range of 1,200-1,350 cm^{-1} (Jackson *et al*., 1995; Kirti and Khora, 2015; Ali *et al*., 2017). Earlier reports have shown an amide A band position in type I collagen

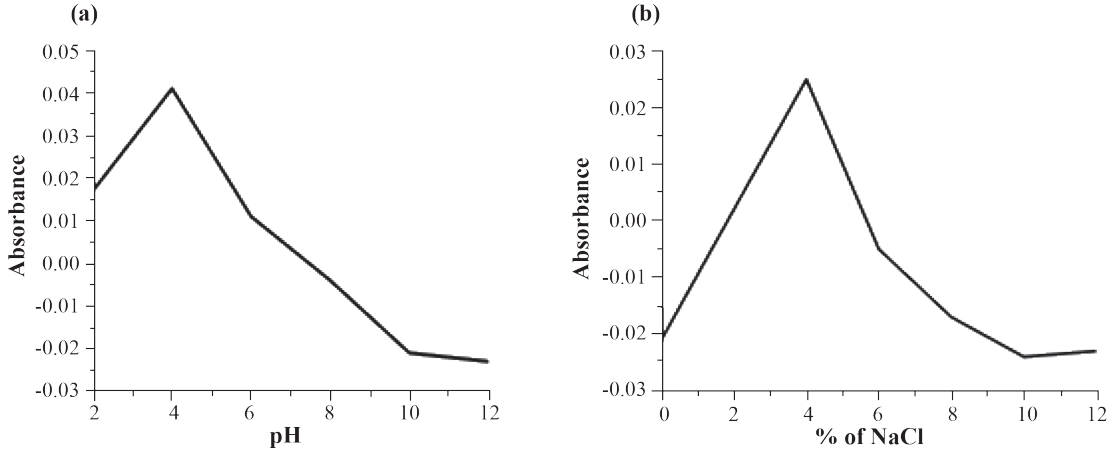

Figure 1. Solubility of ASC isolated from the skin of *Rastrelliger kanagurta* at different pH (a) and NaCl concentrations (b).

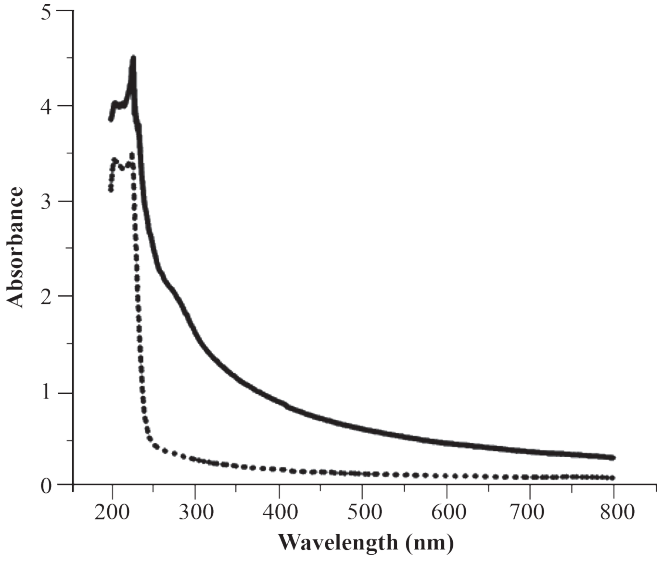

Figure 2. UV-Visible spectra of standard calf skin collagen (represented in solid line) and ASC isolated from the skin of *Rastrelliger kanagurta* (represented in short dot).

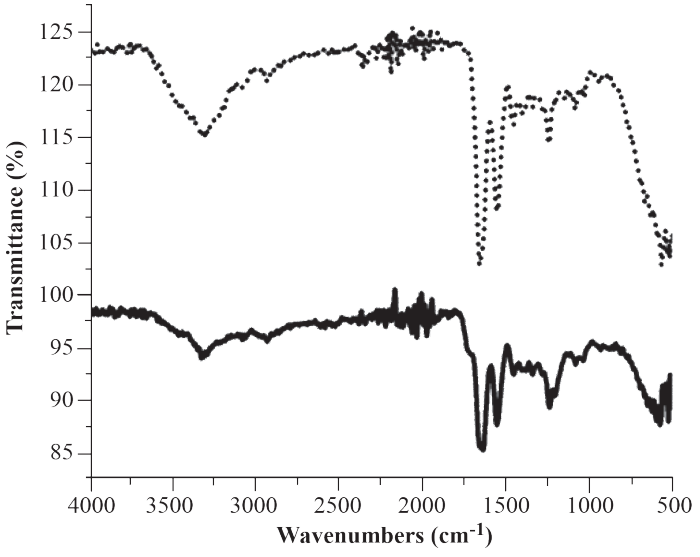

Figure 3. FTIR spectra of standard calf skin collagen (represented in solid line) and ASC isolated from the skin of *Rastrelliger kanagurta* (represented in short dot).

of 3,372 cm^{-1}, which was due to the presence of hydrogen-bonded hydroxyl groups, as well as an amide II band at 1,532 cm^{-1} and an amide III band around 1,350 cm^{-1}, which were indications of the helical structure of collagen (Muyonga *et al*., 2004; Hammed *et al*., 2014; Sripriya and Kumar, 2015). In this study, we also observed similar FTIR bands for isolated ASC. Amide A (3,315 cm^{-1}), amide B (2,926 cm^{-1}), amide I (1,645 cm^{-1}), amide II (1,546 cm^{-1}) and amide III (1,230 cm^{-1}) were identified as major bands in the standard ASC type I calf skin collagen, while amide A (3,310 cm^{-1}), amide B (2,926 cm^{-1}), amide I (1,645 cm^{-1}), amide II (1,544 cm^{-1}) and amide III (1,230 cm^{-1}) were considered as

the major absorption bands in the collagen isolated from Indian mackerel. These data confirmed the helical structure of isolated ASC. In addition, absorption bands around 1,425 cm^{-1} for standard calf skin collagen and for isolated ASC were also found. This corresponded well to pyrrolidine ring vibration of proline and hydroxyproline as explained by Muyonga *et al*. (2004). In the spectrum of collagen there is a distinctive "fingerprint" region between 1,200 and 1,300 cm^{-1} that can be used to identify the particular tripeptide $(Gly\text{-}Pro\text{-}Hyp)_n$ sequence. These results showed the collagen to be preserved in its native conformation during the purification processes (Lin *et al*., 2001; Lin and Liu, 2006).

Thermogravimetric Analysis

The thermogravimetric analysis of the isolated ASC was carried out in the range of 25-700 °C in air at a heating rate of 10 $°C \cdot min^{-1}$ (Figure 4) and compared with standard calf skin ASC. In this analysis actual weight loss was observed in three steps, in the temperature range of 30 ºC-700 ºC. From the graph, it could be inferred that below 100 °C, weight loss occurred mainly due to the loss of water from the protein, while major weight loss occurred at 250 °C, 350 °C and 520 °C for the isolated collagen sample. Complete decomposition occurred at 650 °C. The first decomposition of around 15 % weight loss occurred in isolated ASC at 250 °C; it was due to the larger macromolecules breaking down into smaller ones, 40 % weight loss took place at 343 °C and 70 % weight loss occurred at 520 °C, indicating the production of gaseous elements. Complete decomposition occurred at 650 °C for isolated ASC. A similar result was obtained with standard type I calf skin collagen. Previous reports on collagen isolated from Rohu and Catla fishes have shown a similar pattern, where major decomposition occurred at 535 °C (Pati *et al*., 2010).

SEM analysis of ASC

The SEM image indicated that the isolated collagen was fibrillar in nature and densely packed. The diameter of collagen fibers was measured on micrographs and found to be 10 µm, as shown in Figure 5. The porous structure of collagen was clearly visible. The fiber network of collagen can be cross-linked by hydrogen bonds, electrostatic bonds, hydrophobic interactions, entropic and dispersion forces (Kirti and Khora, 2015; Suarez *et al*., 2015). The isolated sample was undoubtedly pure and could be taken directly for the study.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight of the mackerel collagen was determined by using SDS-PAGE with reference to type I calf skin collagen, as shown in Figure 6. The molecular weights of the four bands, namely α1, α2, β and γ chains, were found to be 149.59 kDa, 136.7 kDa, 214.49 kDa and 256.85 kDa, respectively. The electrophoretic patterns revealed that the isolated ASC consists of high proportions of α chains and high molecular weight components (β chains) and a small quantity of γ-chain components (Yamaguchi *et al*., 1976; Veeruraj *et al*., 2013). The presence of the β component confirmed that the collagen contains more intermolecular cross-links. The presence of the γ component indicated that the three chains of collagen are intra-molecularly cross-linked, which assists the renaturing of native collagen (Lewis and Piez, 1964; Muralidharan *et al*., 2013). The skin collagen of bigeye snapper, brownbanded bamboo shark, ocellate puffer fish, Nile perch, seabream, back drum, sheepshead seabream, Spanish mackerel, brown-backed toadfish, seabass, bighead carp, ornate threadfin bream, black carp, rohu, striped catfish and amur sturgeon all contained two α chains (α1 and α2), β and γ components (Nagai *et al*., 2002; Ogawa *et al*., 2003; Muyonga *et al*., 2004; Jongjareonrak *et al*., 2005b; Senaratne *et al*., 2006; Kittiphattanabawon *et al*., 2010; Singh *et al*., 2011). The presence of two identical α chains indicates that the extracted collagen belongs to type I (Hema *et al*., 2013; Suarez *et al*., 2015; Chen *et al*., 2016).

Wound-healing sample preparation

The collagen gels were prepared along with the major constituents found in papaya leaf, including caffeic acid, coumaric acid, quercetin and oleic acid. The tests were carried out in one-to-one

combination as well as in mixed combinations. Glycerol was used for the preparation of collagen gel because it is stabilizes the triple helical structure of collagen and decreases the rate of collagen denaturation (Na, 1986). Clinical studies have shown that glycerin, at a high concentration, creates a bacteriostatic environment, which reduces the number of microorganisms in the wound. This is a definite benefit when treating wounds and results in better healing outcomes (Stout and Mckessor, 2012).

MTT Assay

The *in vitro* cytotoxicity of collagen was investigated by using mouse embryonic fibroblast NIH3T3 cells by MTT assay. The cytotoxicity study is very important for wound healing applications. Cell attachment, proliferation and differentiation depends on the nature of the matrix and cell type (Sripriya and Kumar, 2015). The cytotoxicity results of prepared collagen gels are shown as IC_{50}

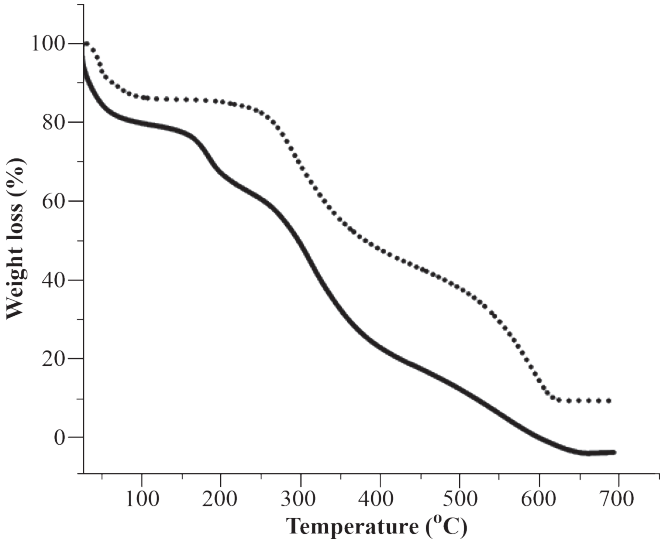

Figure 4. Thermogravimetric analysis of standard calf skin collagen (represented in solid line) and ASC isolated from the skin of *Rastrelliger kanagurta* (represented in short dot).

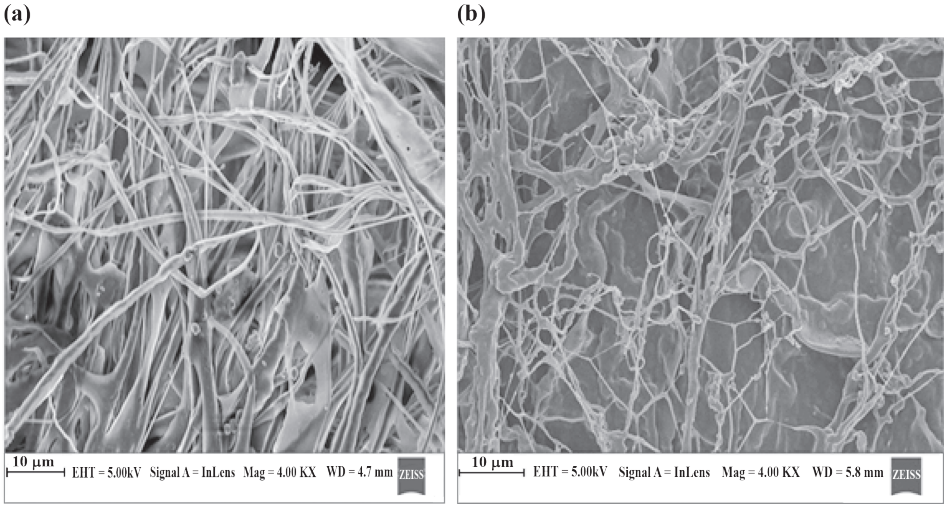

Figure 5. SEM images of standard calf skin collagen (a), ASC isolated from the skin (b) of *Rastrelliger kanagurta*.

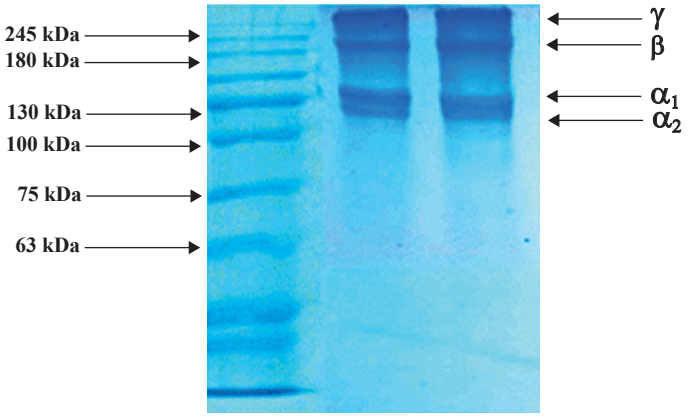

Figure 6. SDS–polyacrylamide gel electrophoresis of ASC. Lane 1 contains high molecular weight markers of 10-245 kDa. Lane 2: standard type I calf skin collagen. Lane 3: ASC isolated from the skin of *Rastrelliger kanagurta*.

values and listed in Table 3. In this study all the test compounds showed IC_{50} value <200 µg·mL^{-1} except samples 5 and 6. Cytotoxicity test results showed that samples less than 50 µg·mL^{-1} resulted in more than 50 % cell viability. Based on these results, 25 µg of test compounds were used for further study, as this level was not toxic to the cell line.

Scratch assay

During the wound healing process, the migration of keratinocyte cells accelerates the re-epithelialization process and promotes wound closure. A scratch assay was used to simulate wound closure where a "wound gap" was created by scratching a cell monolayer. The "healing" of the gap took place by cell migration and growth towards the center of the gap, and was monitored and frequently measured. In the present study the effects of MCPs on the healing process were investigated by using *in vitro* scratch assay with NIH3T3 cells, as shown in Figure 7. The scratch closure rate was calculated at 24 h. Wound migration was determined by using image J software. The different test compounds (25 µg) were used for scratch assays. The concentration of test compounds was selected based on the MTT assay. The migratory nature of fibroblasts was observed by microscopic examination. The distance of cell migration was measured quantitatively for 24 h under 40X magnification, as it is directly proportional to the wound closure mechanism. The cell migration was rapid in sample 4 compared to other test compounds, with 100 % migration (Figure 7 and 8). Sample 4 (S4) contained collagen along with quercetin, which enhances the wound healing process. This is probably because quercetin enhances the production of collagen and fibronectin, which act synergistically to speed the closure of the wound (Suntar *et al*., 2010; Krishnappa *et al*., 2016).

Table 3. IC_{50} values of prepared collagen gels.

Test compound	IC_{50} (µg±SD)
Sample 1 (S1)	148.43±6.24
Sample 2 (S2)	121.09±8.42
Sample 3 (S3)	54.68±2.01
Sample 4 (S4)	59.66±2.01
Sample 5 (S5)	259.80±21.06
Sample 6 (S6)	233.23±20.05

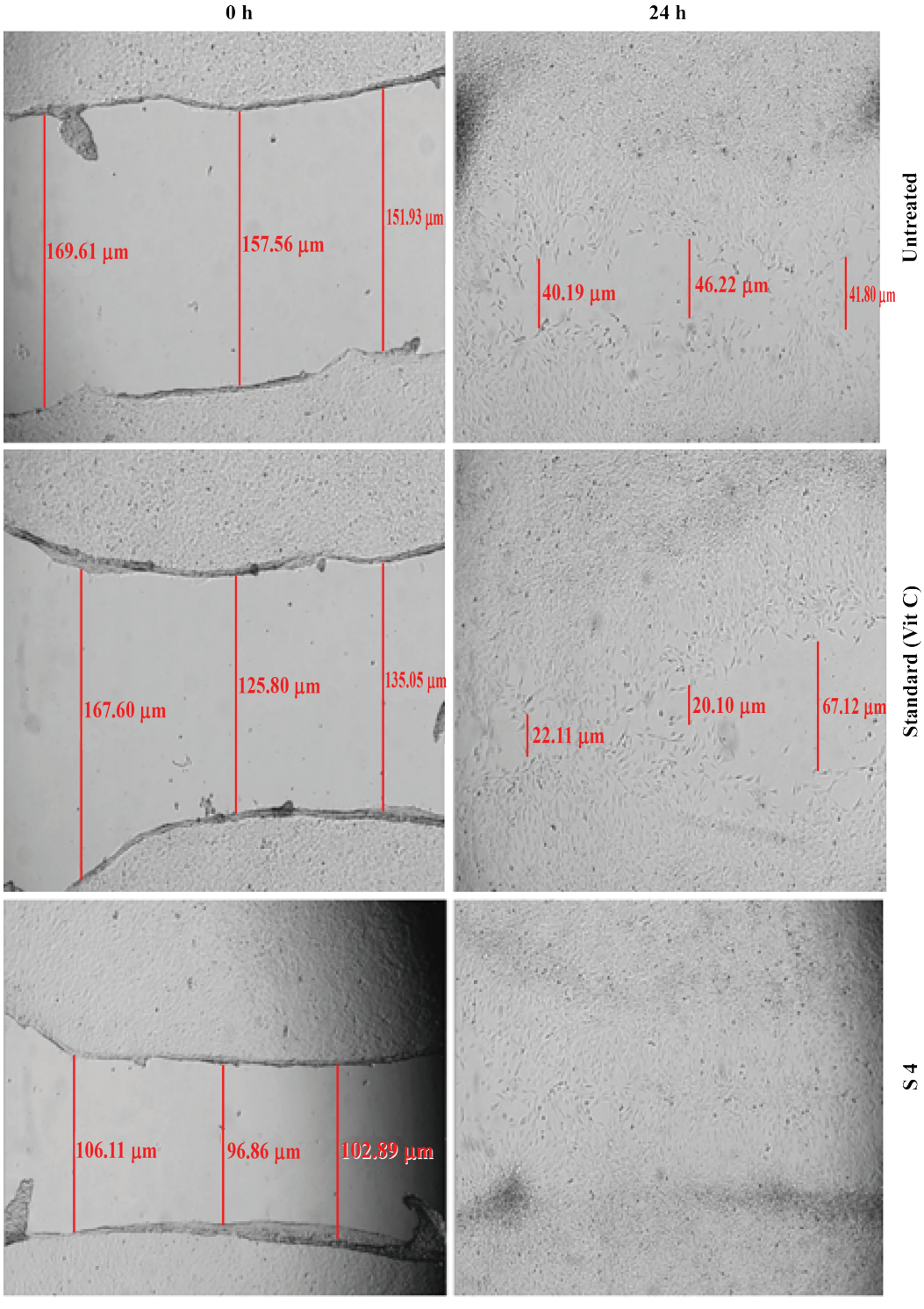

Figure 7. Photographs of scratch assays on NIH3T3 cells at zero and 24 h under 40X magnification.

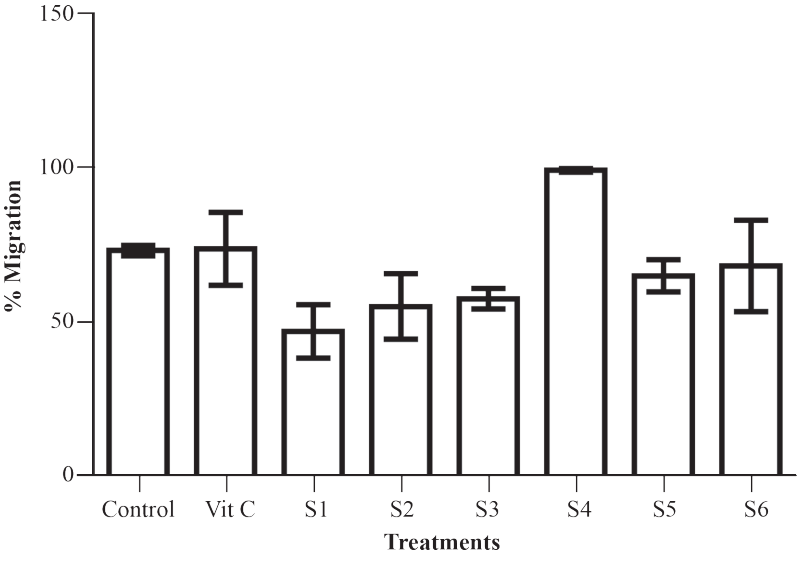

Figure 8. Wound closure as measured by fibroblast percentage migration in scratch assays of NIH3T3 cells. Columns represent mean±SD from three replicates.

CONCLUSION

Acid-soluble collagen was extracted and characterized from the skin of Indian mackerel. The yield of collagen obtained from the skin was found to be 3.57 %. The amount of protein was found to be 34.68 $mg \cdot g^{-1}$. Confirmatory characterization of collagen was carried out by using SDS-PAGE, UV, FTIR, TGA and SEM. The maximum solubility of ASC was found to be at pH 4 and 4% NaCl. The present study investigated the effect of collagen gel along with different papaya leaf constituents on *in vitro* wound healing assays using the NIH3T3 cell line. Results of *in vitro* cytotoxicity tests showed that prepared collagen gel did not induce a significant cytotoxic effect and could be utilized as a safe material for preparing biocompatible collagen-based gels. The most effective migration was observed in sample 4 (collagen+quercetin) at 25 $\mu g \cdot mL^{-1}$ concentration, with 100 % migration of cells and resulting in complete wound closure. As a result, collagen extracted from the skin of Indian mackerel can be used as an effective biomaterial for wound dressing. The marine material can be used as an alternative to bovine or human collagen in selected biomedical applications.

ACKNOWLEDGEMENTS

The authors would like to acknowledge funding from a NFSC fellowship (Id No. RGNF-2017-18-SC-KAR-41024). They also acknowledge DST PURSE and University Science Instrumentation Centre (USIC), Mangalore University for providing the SEM, TGA, UV spectral analysis and FTIR facilities.

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