

Rejuvenation of Burn Infection Through Medicinal Plants Incorporated Collagen

Gunasheela.N, Manovina.M

ABSTRACT: Collagen is a major natural constituent of connective tissue and structural protein of any organ which has the applicability as biomaterial drug delivery system. Fish collagen has gained increasing interest as the alternative for mammalian counterpart. Collagen acts as regenerative biomaterial for treating burn infection. Burned tissue or skin gets easily infected because the skin has lost its ability to protect the underlying tissues from microorganisms. Gram-positive bacteria are some of the first to colonize burns, followed quickly by gram-negative. Two bacterial species, Methicillin-Resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* will be examined in depth in this work as they are two of the most prevalent infective agents. These two species have proven particularly difficult to treat because they possess a large number of virulence factors and antimicrobial resistance genes. A plant with multi-potent pharmaceutical activities offers better treatment option. The extracts made from *Euphorbia hirta* and *Triphala* can speed the healing process by stimulating blood flow to the skin. In the present research work fish collagen was extracted from scales and the molecular weight was determined. The plant extract were prepared and unified with collagen membrane. The antimicrobial activity of *Euphorbia hirta* coated collagen membrane was performed against wound causing pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* for eliminating burn infection along with wound healing property.

Keywords: fish collagen, burn infection, *Euphorbia hirta*, microorganisms, wound healing activity.

1 INTRODUCTION

The expanding medical applications of biomaterials and connective tissue research challenged academically oriented scientists and commercial research laboratories to focus their studies on collagen. At the same time, medical-grade collagen became easier to obtain, the processing technology improved, and new collagen products were successfully placed on the market. Collagen is a major natural constituent of connective tissue and structural protein of any organ which has the applicability as biomaterial drug delivery system. Collagen consists of amino acids bound together to form triple-helices to form elongated fibrils. Collagen can be widely used as biomaterials in various forms like threads, sutures, scaffolds, membranes, films and also as fillings in dental surgery. Fish collagen is the Type I Collagen that has gained increasing interest as the alternative for mammalian counterpart. Fish skin accounts for nearly 6% of the live weight of fish especially in seer fish. It forms a major portion of skin and is easily obtainable and abundant. It is considered a significant source of highly soluble collagen. Skin parts are considered as waste product during filleting process. If it is used to obtain collagen, it will definitely increase the demand of the skin. Waste skin products can be widely used as a beneficial one which aids in reducing environmental pollution. It also helps to keep the wound sterile, because of its natural ability to fight against infection. When collagen is used as a burn dressing, healthy granulation tissue is able to form very quickly over the burn, helping it to heal rapidly. Bacteria and fungi are the most common pathogens of burn wounds. These microbes form multi-species bio films on burn wounds within 48 – 72 hours of injury. Organisms originate from the patient's own

skin, gut and respiratory flora, as well as from contact with contaminated environments. Gram-positive bacteria are some of the first to colonize burns, followed quickly by gram-negative. Two bacterial species, methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two of the most prevalent infective agents. These two species have proven particularly difficult to treat because they possess a large number of virulence factors and antimicrobial resistance genes. Due to certain disadvantages alternative method for treating wound infection was developed. The discovery of collagen act as biomaterial for wound healing activity. A plant with multi-potent pharmaceutical activities offers better treatment option. *Euphorbia hirta* is a pan tropical weed, native to India. It is a hairy herb that grows in open grasslands, roadsides and pathways. It is widely used as a medicinal herb. All parts of the plant are effective in their own way, and are used for specific ailments. The leaves, flowers, and sap from this herbaceous plant, which is also commonly called an "asthma weed", all are used in different ways and in different forms, depending on the ailment at hand. The extracts made from *Euphorbia hirta* can be applied directly to the skin on boils, wounds, rashes, burns, and other marks. This extract can speed the healing process by stimulating blood flow to the skin, stimulate the regenerative growth of new cells, and even contribute its antioxidant capacity to promote healthy looking skin. It can eliminate signs of aging and reduce the appearance of wrinkles and sun damage.

Materials and methods:

Sample collection

Seer fish skin waste was collected from Ukkadam fish market, Coimbatore and it was refrigerated at 4°C.

Collagen Extraction

The fish skin collected from waste was made free from attached muscle and scales using sharp knife. After cleaning the skin, 50 g of skin was chopped into fine small pieces (0.5 x 0.5 cm). Chopped skin was refrigerated at 4°C.

Removing non collagenous tissues

The skin was treated with 1 litre of 0.1M of NaOH (pH 12) for 24 hours to remove non collagenous substance. Then, the solution containing the skin was gently stirred for 5 minutes. The resulting solution was refrigerated for 24hrs and filtered. Then the skin was washed thrice thoroughly to reach its neutral pH. After each wash, pH was measured. The skin was treated with 10% butyl alcohol (1:10) for 48 hours to remove fat tissue from the skin. The solution containing skin was filtered and again treated with 10% butyl alcohol. Finally the solution was refrigerated for 24hrs.

Solubilisation of collagen

The complete removal of fat occurs after 2 days of alcohol suspension, the solution was filtered and the skin was washed four times thoroughly using cold distilled water. Once the washing was overed the skin was suspended in 1 litre of 0.5% acetic acid for 3 days with gentle agitation at 4°C to solubilise the fibril collagen into tropocollagen which is the subunit of collagen fibril. After 3 days of acetic acid suspension, the solution was filtered by using double filter papers and the filtrate was obtained.

Concentration of collagen

The extracted collagen was placed in oven at 30°C and the solution was evaporated and the final weight of collagen was measured.

Characterization of collagen

SDS-PAGE is performed using the discontinuous Tris-HCl/glycine buffer system with 7.5% resolving gel and 5% stacking gel. After electrophoresis, the gel is stained for 20 minutes with 0.1% Coomassie Brilliant Blue dissolved in distilled water, methanol and acetic acid (9:9:2), and then the same is destained using a solution of distilled water, methanol and acetic acid (8:1:1). Protein molecular mass was calculated in comparison with known protein molecular weight such as high range molecular weight marker is used.

Preparation of extracts from *Euphorbia hirta*

Euphorbia hirta leaves were collected from road side area of Coimbatore. The leaves were washed thoroughly and dried under shade for 20-25 days. After drying; the leaves were grinded in to coarse powder in order to obtain extracts.

Preparation of aqueous extract

Decoction was prepared by boiling 10g of fine powder with 100 mL distilled water for 15 minutes in a stainless steel

casserole. The crude aqueous extract was filtered through muslin cloth and concentrated. The concentrated aqueous extract was brown in color with a pH of 5.02. The filtrate was evaporated to dryness using a rotary evaporator.

Preparation of ethanolic extract

20 g of the fine powder were weighed and dispensed into 250 ml of ethanol (95%) in a conical flask. This was covered, shaken every 30 min for 6 h and then allowed to stand for about 48 h. The solution was subsequently shaken and filtered using what man filter paper. The filtrate was evaporated to dryness using a rotary evaporator. Incorporation of aqueous and ethanolic extracts into collagen film

The aqueous and ethanolic extracts of *Euphorbia hirta* was dissolved in 10% Dimethyl Sulfoxide. 1ml of prepared solution was poured into the culture dishes containing collagen membrane. The culture dishes were incubated for 30 minutes.

Collection of Test organism

Microorganisms like *Staphylococcus aureus* and *Pseudomonas aeruginosa* was collected from Sri Ramakrishna Hospital, Coimbatore.

Confirmation of organisms by microscopic examination and cultural characteristics:

The cultures were checked for purity by gram staining and *Staphylococcus aureus* was cultured in Mannitol Salt Agar and *Pseudomonas aeruginosa* was cultured in Cetrinide Agar.

Confirmation of organism by biochemical tests:

The cultures were confirmed by biochemical characterization.

Test organism preparation

The test organisms (*S. aureus*, *P. aeruginosa*) were inoculated in 50ml of Soya bean casein digest broth and incubated overnight at 37°C in a rotary shaker. 2ml of overnight cultures (*S.aureus*, *P.aeruginosa*) were inoculated in 100ml of Soya bean casein digest broth and incubated for 4 to 5 hours (late exponential phase). After incubation the bacterial cells were harvested by centrifuging at 20,000rpm for 10 minutes at 4°C and washed thrice with 60ml of 0.1 M NaCl per phosphate buffer saline and re-suspended in the same buffer. The cell suspension was aspirated and expelled with syringe with 25 gauge needle. The bacterial suspension in phosphate buffer saline was filtered through membrane filters (8µm) to remove clusters and the filtrate was adjusted to get the optical density of 1.0 using spectrophotometer at 540nm with same buffer.

Preparation of bacterial cell suspension

3 ml of prepared bacterial cell suspension was added to the culture dishes containing aqueous and ethanolic extract incorporated collagen membrane and plain collagen membrane and incubated at 37°C in rotary shaker for four hours. After incubation the collagen membranes were

rinsed several times (eight to ten times) with buffer. The membranes were treated with 2.5% glutaraldehyde (2.5 ml glutaraldehyde in NaCl/phosphate buffer saline) and incubated for 5 minutes. The membranes were finally rinsed with sterile distilled water and dried at room temperature.

Antibacterial activity of Euphorbia hirta coated collagen membrane and Plain collagen membrane

The antibacterial activity of aqueous and ethanolic extract incorporated collagen and plain collagen membrane was determined against wound infection causing pathogens like Staphylococcus aureus and Pseudomonas aeruginosa. The number of bacteria adhered to the collagen membrane was counted under bright field microscope.



Result and Discussion

Sample collection

Seer fish skin waste as collected and stored at 4°C for future use.

Figure 1: Seer fish skin after filleting.



Extraction of collagen

The collagen was extracted from 50gms of seer fish skin using acid-solubilised collagen method. It is very important for the processes to be done at low temperature in order to reduce heat denaturation on the extracted collagen and to preserve their native structure. The procedure in this study was done at 4°C temperature. Acetic acid is usually used and the collagen obtained using acid extraction is termed as acid soluble collagen.

Fish skin preparation

The cleaned skin was cutted and used for the extraction process. The purpose of cutting the skin would make the collagen extraction to become easy.

Removing non collagenous tissues

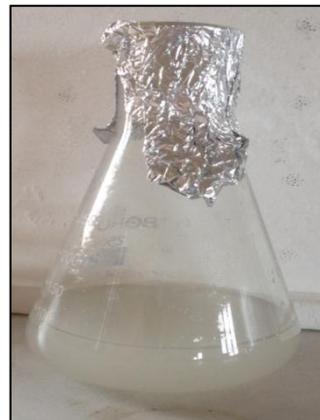
The use NaOH for skin treatment is to remove pigments present in the skin and also it makes the skin to become very loose in nature. Whereas the role of butyl alcohol is to remove fat content from skin.

Figure 2: NaOH and butyl alcohol treated skin

Solubilisation of collagen

The acetic acid treatment will solubilise the fibril collagen into tropocollagen which is the subunit of collagen fibril. After the treatment with acetic acid the resulting solution was filtered and then collagen was obtained.

Figure 3: Collagen extract



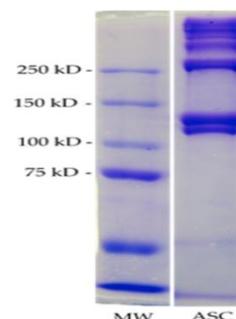
Concentration of collagen

The final concentration of extracted collagen was 0.104g/ml. The total amount of collagen which was extracted from 50g of fish skin was 0.8314g which represents 1.66% from the used skin.

Characterisation of collagen

The SDS-PAGE technique separates macromolecules such as nucleic acids and protein fragments according to their molecular weight or size, based on differences in electrophoretic mobility under an applied electrical field.

Figure 4. SDS-PAGE patterns for seer fish skin extracts show the characteristic bands of collagen



Preparation of extracts from Euphorbia hirta

The aqueous and ethanolic extracts were prepared and dissolved in 10%DMSO and stored for future use.

Figure 5: The aqueous and ethanolic extracts of Euphorbia hirta



Incorporation of Aqueous and Ethanolic extracts into collagen film The aqueous and ethanolic extract incorporated collagen film was dried and organisms were added to check its activity.

Figure 6: The aqueous and ethanolic extract incorporated collagen film



Collection of Test organism

Staphylococcus aureus and Pseudomonas aeruginosa were collected and subcultured for the further use.

Confirmation of organisms by microscopic examination and cultural characteristics

The Staphylococcus aureus was found to be in purple color gram positive cocci in clusters and the Pseudomonas aeruginosa was identified by pink color gram negative rods under microscopic view.

Figure 7. Staphylococcus aureus shows the yellow color pigmented colonies on Mannitol salt Agar and Pseudomonas aeruginosa shows the green pigmented colonies on cetrimide agar



Confirmation of organism

The Biochemical characteristics of Staphylococcus aureus, Pseudomonas aeruginosa was shown.

Table 1: Biochemical characteristics

Biochemical Reaction	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>
Indole test	-	-
Methyl Red Test	-	-
Voges Proskauer Test	-	-
Citrate Utilization Test	-	+
Catalase Test	+	+
Oxidase Test	+	+
Coagulase Test	+	-
Nitrate Test	+	-
Glucose Fermentation	acid production	acid production
Mannitol Fermentation	Yellow pigmented colonies	-
Triple Sugar Iron Agar	Acid bud, Alkaline slant and H ₂ S production	Alkaline bud, Alkaline slant and No H ₂ S production

Preparation of bacterial cell suspension

The test organisms were incorporated in to the collagen film and after incubation the excess cultures were removed and its antimicrobial activity was determined.

Antibacterial activity of *Euphorbia hirta* coated collagen membrane and Plain collagen membrane

The number of organisms adhering to the *Euphorbia hirta* coated collagen membrane and Plain collagen membrane.

Table 3: calculation of number of organisms adhering to the Euphorbia hirta coated collagen membrane and Plain collagen membrane.

Test organisms	Number of bacteria		
	Plain collagen membrane	Euphorbia hirta incorporated collagen membrane	
		Aqueous extract	Ethanollic extract
Staphylococcus aureus	$56.3 \pm 3.5 \times 10^5$	50 ± 2	64 ± 2
Pseudomonas aeruginosa	$31.3 \pm 3.5 \times 10^4$	123 ± 3	115 ± 3

Conclusion

The seer fish skin acts as an abundant source of collagen which has wide applications in Medical and Pharmaceutical areas. Nowadays the use of biomaterials has been increased; unconvincingly collagen also serves as the essential biomaterial for their tissue regenerating ability and its availability in nature. Even though it has a wide application it could not control the growth of microorganisms for this purpose the natural medicinal plants have been suggested because of its antibiotic resistance character. The usage of such medicinal plant would reduce the the growth of microrganisms. For this above reasons Euphorbia hirta was selected as natural wound healer. The extracted collagen was unified with the leaf extracts of Euphorbia hirta to check its antibacterial activity against the burn infection causing pathogens such as Staphylococcus aureus and Pseudomonas aeruginosa, because these two pathogens was estimated as primary and major contaminants of burn wound ,as they shows high antimicrobial resistance towards certain antibiotics . This present research work conveys that the collagen along with Euphorbia hirta was found to be the excellent combination for reducing the bacterial infection along with its wound healing ability.

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