

Preparation of Collagen Particles from Fish Waste Infused with Cassia Auriculata

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The human skin encompasses a characteristic capacity to advance the self-regeneration after harm; this capacity can be compromised beneath particular conditions, like extensive skin loss, constant wounds, deep burns, non-healing ulcers and diabetes. Improper healing can lead the wound to enter in a constant state, which increments the chance of disease to chronic state; it affects the patient health and his/her quality of life. Due to growing concerns about unhealthy consequences of chemicals in the health care system, the interest towards natural and herbal substances has been growing every day. The plant Cassia auriculata is widely used medicinal plant in India and also popular in Indigenous system of medicines like Ayurveda and siddha. The collagen was successfully extracted from the *Cattla Cattla* fish scales using acid solubilizing collagen extraction method and the collagen was infused with plant extract of Cassia auriculata. The characterisation such as protein estimation (Lowry method), solubility (pH 1, 3, 5, 7, 9, 11 and 13), UV visible and FTIR was performed for collagen and phytochemical analysis (Alkaloids, Flavonoids, Saponins, Tannins, Phenol, Terpenoids, Glycosides and Steroids) anti-bacterial (*E. coli* and *S. aureus*) and anti-inflammatory test in different concentration (25mg, 50mg, 100mg/ml) was performed for the plant extracts and collagen infused with plant extracts. The result of the present study was confirmed that final product prepared using collagen with plant extract can be used to prepare the dressing material such as gels, ointments, bio-films, powdered flakes etc.

Keywords: Collagen; Cassia auriculata; Diabetic Foot Ulcer; Marine Source; Phytochemicals.

Wound healing is a complex progression that takes place in skin after it has been damaged. Hemostasis, inflammation, proliferation and maturation are the four stages of wound healing¹. In hemostasis phase, platelets aggregate and stick to the epithelial wall of blood vessels, where fibrin strands begin to adhere to the blood and transformed for the release of prothrombin. Platelets and blood cells are trapped in the wound area and forming a clots. In damaged cells, germs

and bacteria are evacuated from the wound region during the inflammatory phase. Swelling, heat, discomfort and redness are frequent during this stage of wound healing and can be caused by white blood cells, growth hormones, nutrients and enzymes. In proliferation phase, new collagen and extracellular matrix-based tissues are formed in the injured area. A new network of blood vessels is being built to ensure that the damaged area receives enough oxygen and nutrients. The maturation phase

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of wound healing is last stage, when collagen is transformed from type III to type I, the wound is completely healed. Healed wound have a lower tensile strength than normal skin, with just 80% of the tensile strength of unwounded skin²

Physiological changes in tissues and cell induced wound healing get delayed and difficult to manage in diabetics, as well as having their impact. Diabetes affects the healing of both acute and chronic wounds³ Factors both external and internal to the wound and its biology impede wound healing in diabetes mellitus. Thickening of the capillary and arteriole membranes is common in diabetics, leading to slow wound healing and the formation of persistent ulcers⁴.

Collagen is a selective organic macromolecule with a triple helix structures that make up the larger part of the extracellular dermal network (ECM). In both vertebrate and invertebrate creatures, collagen is the foremost plenteous mammalian protein and the major basic strong protein of the extracellular matrix. Collagen is found in sinewy connective tissues, the dermis of the skin, corneas, cartilages, bones and sclera, where it plays a vital basic part in tissues and organs by giving malleable quality and flexibility^{5,6}. Collagen type I accounts for 70% of collagen in the skin, with type III accounting for 10% and collagen types IV, V, VI, and VII accounting for trace amounts. Collagen's main purpose in connective tissue is to act as a scaffold, which it does primarily in its type I, II, and III forms. Type III formed first during the early stages of wound healing, with the amount of type I increasing as scar formation develops and it is rebuilt^{7,6}. Diabetes patients have poor biomechanical qualities, such as increased skin stiffness and decreased skin elasticity. Collagen synthesis and breakdown are out of balance, resulting in these characteristics⁵. External collagen sources, such as plant-based collagen, marine collagen and so on, can be used to boost tissue regeneration potency when collagen production is lacking. The abundance of structurally and physiologically active chemicals found in marine species is surprising and plentiful. Fish, seaweeds, sponges and jellyfish are all sources of marine collagen. It has several advantages over mammalian collagen, including as it can be extracted easily, the fact that it is water-soluble, it is free of animal disease and infections, it has

better chemical and physical durability and also it is readily available. It has a wide range of applications due to its biocompatibility, biodegradability, ease of supply and adaptability⁸.

In 21st century, home grown drugs are being favoured generally compared to cutting edge medication due to their security, social adequacy, viability and less antagonistic impacts. Conventional herbs have a long history of practice and are considered to be more secure than engineered drugs. Plant and plant based compounds have been utilized with changing victory to diminish, avoid illnesses and diseases in all the time⁹. One such a plant *Cassia auriculata* commonly known as "avaram" is a shrub that belongs to the Caesalpiniaceae family. The individual parts of the plant can be used for the treatment of various disease and disorders in humans. The plant contains several biological properties like hepato protective, anticancer, antioxidant, antidiabetic, anti-inflammatory and antimicrobial properties¹⁰. It has phytochemicals like alkaloids, tannins, saponins, phenolics and terpenoids exhibit excellent anti-inflammatory activity. Restorative properties of its flower have antioxidant and antimicrobial activity was contributed by nearness of its phytoconstituents. The flavonoids shown in it act as cancer prevention agents which give assurance against free radicals that harm cells, tissues additionally the tannins progress the wound recuperating¹¹.

Wound dressing derived from common body components may be beneficial to progress biocompatibility and to maintain a strategic distance from conditions like aggravation or rejections. The most objective of employing a natural wound dressing is to improve wound patching by avoiding bacterial infection and to speed up the tissue recovery. Wound dressing materials should have basic properties like adaptability, durability, gas penetrability and capacity to avoid water loss. Collagen as base fabric offers a few points of interest, which has well recorded basic structural, physical and chemical properties. Hence, the present work focussed on the preparation of collagen from marine source and it can be incorporated with *Cassia auriculata*, which can be used as dressing material for diabetic wound.

MATERIALS AND METHODS

Preparation of Fish sample for collagen extraction

Waste parts of *Cattla Cattla* fish (scales) was washed in water and shade dried for 6-7 days until they are completely dried. Then the samples were ground into small pieces by using a blender. Pre-treatment was conducted, to make sure all the unwanted materials like unground scales were removed. Therefore there will be no unwanted materials that will affect the collagen and make sure that the collagen was completely extracted without any remote materials.

The fish scales were soaked in 0.1 M sodium hydroxide (NaOH) preparation with proportion of 1:8 (w/v) for 6 hrs and kept in stirrer for the expulsion of non-collagenous protein. The NaOH solution was changed for each 3 hrs washed completely with cold refined water until the flushed water ended up neutral (pH 7) as measured by using pH meter¹². Fish scales were soaked in 0.5 M Ethylene Diamine Tetra Acetic acid (EDTA) at pH 7.4 for 24 hrs with persistent mixing. The solution was changed for each 12 hrs. Then, it was washed with refined water for 3 times¹³.

The collagen was extracted using acid soluble collagen extraction method.

- The samples were undergone acid soluble collagen (ASC) extraction process by using 0.5 M acetic acid with the sample in solution ratio 1:10 (w/v) for 24 hrs with continuous stirring.
- The extracts were centrifuged at 10,000 x g for 30 mins at 4°C and the supernatant was separated. The residues then were re-extracted with 0.5 M acetic acid with sample to solution ratio of 1:10 (w/v) for 12 hrs and centrifuged at 10,000 x g for 30 mins at 4°C.
- Both supernatants were combined and sodium chloride (NaCl) was included to salt out until the ultimate concentration of the supernatant was 0.7 M for the precipitation to happen.
- The supernatant was centrifuged once more at 2,500 x g to get the precipitate. The precipitates were lyophilised¹⁴

Preparation of aqueous extract of *Cassia auriculata*

The *Cassia auriculata* flowers were collected and shade dried for 3-4 days. From

this the unwanted parts like stems and leaves are removed.

- The dried flowers were made into partial pieces using a blender.
- One gram (1g) of blended flowers were mixed with 20 mL of water and kept in shaking incubator at 50-60 rpm for 24 hrs.
- The mixture was then filtered through the Whatman No.1 filter paper to ensure that no particles were present in the solution and the extract was collected (Fig -1).
- The filtered solution was again filtered using syringe filter¹⁵.

Infusion of collagen with *Cassia auriculata*

- The prepared collagen from fish scales and flower extract from *Cassia auriculata* was combined together using cross linker.
- Using 0.05M acetic acid (in the ratio 1:10) collagen solution was prepared
- The solution was agitated using magnetic stirrer for 1h and kept for 24 h incubation at 4°C.
- The solution was then filtered and homogenized.
- 25µg/ml, 50µg/ml, 100µg/ml of plant extracts were added to 20ml of collagen solution and mixed well.
- Varying quantities of ethylene glycol (cross linker) were added (0.5ml-2.5ml).
- Solution was poured in plates and it was air dried or freeze dried (Fig-2).
- The prepared collagen samples were stored for further analysis¹¹

Estimation of protein by Lowry's method

Reagent A: It consists of aqueous solution of 0.4% potassium-sodium tartrate, 10% Na₂CO₃, and 0.5 M NaOH.

Reagent B: It consist of an aqueous solution of 2% potassium-sodium tartrate, 3% CuSO₄, 5 H₂O and 0.1 M NaOH. Reagents A and B are stable for 1 month at room temperature. The Folin-Ciocalteu reagent was diluted with water (1:15), the resulting solution being 0.15 N.

- Incubation of collagen samples in alkaline solution at high temperature (50°C) leads to the unfolding of the collagen rigid triple helix (the melting temperature of collagens being - 40-43°C). In this way, the collagen polypeptide spines can form complexes with Cu²⁺ more promptly and decrease Folin's reagent. 200 µl of collagen solutions were incubated with 180 µl of reagent A and 20 µl of reagent B

at 50°C for 20 min. After cooling at room temperature, 600 µl of diluted Folin-Ciocalteu reagents were included and forcefully shaken and the samples were incubated at 50°C for 20 min. The absorbance of the samples was read at 650 nm against the reference after cooling in room temperature. BSA was utilized as standard.

Solubility of collagen at different pH

The dissolvability of the collagens at the diverse pH and NaCl concentrations were decided by the method of Montero *et al.*,¹⁶ with a slight alteration. Crude collagen samples were broken down using 0.5 M acidic acid to get a final concentration of 3 mg/mL and the blends were mixed at 4°C for 24 h. At that point, the blends were centrifuged at 5000 g for 15 min at 4°C. The crude solutions (8 mL) were transferred to 50 mL centrifuge tube. The pH was balanced with either 6 M NaOH or 6 M HCl to get the final pH extending from 1, 3, 5, 7, 9, 11 and 13. At that point, the volume of solution was made up to 10 mL by deionized water and the solution pH was balanced to the same pH as the collagen solution. The solution was centrifuged at 15000 g for 30 min at 4°C. Finally the protein substance within the supernatant was measured.

*Percentage of Relative solubility = Estimated protein content / 3 mg (Initial volume taken) × 100.

Phytochemical analysis of plant extract

Alkaloids

In a test tube 0.5ml of extract, few drops of Meyer's reagent were added. White precipitate indicates the presence of Alkaloids.

Flavonoids

In a test tube 0.5ml of extract few drops of 20%NaOH was added. Intense yellow colour indicates the presence of Flavonoids.

Saponins

In a small test tube, 0.5ml of extract, 2ml of distilled water was added and shaken vigorously. Formation of bubbles shows the presence of saponins.

Tannins

In a test tube, 0.5ml of extract few drops of alcoholic FeCl₃ was added. Formation of brownish blue or black colour indicates the presence Tannins.

Phenol

In a test tube, 0.5ml of extract few drops of 5% FeCl₃ was added. Formation of blue-black

colour indicates the presence of phenol.

Terpenoids

In a test tube 0.5ml of extract, 0.5ml chloroform and few drops of H₂SO₄ was added. Reddish brown colour indicates the presence of terpenoids.

Glycosides

0.5ml of extract, 0.5ml glacial acetic acid and few drops of FeCl₃ was added. Formation of brown ring indicates the presence of glycosides.

Steroids

In a test tube 0.5ml of extract, 0.5ml chloroform and a few drops of conc.H₂SO₄ were added. Chloroform layer appears red while acid shows Fluorescence.

Antibacterial activity

The anti- bacterial assay was carried out for both plant extract and collagen infused plant extract. The bacterial strains of one gram negative bacteria (*Escherichia coli*) and one gram positive bacteria (*Staphylococcus aureus*) were standardized to 0.5 McFarland to obtain 10⁸ cells/ml. Amoxicillin was used as a positive control whereas water was used as negative controls.

Anti-inflammatory assay

BSA Inhibition Method: The anti-inflammatory activity was carried out by Bovine Serum album method. The percentage inhibition was calculated for both plant extract and plant extract infused collagen.

$$**\% \text{ Anti-Denaturation Activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

$$* \% \text{ Anti-Denaturation Activity} = \% \text{ Inhibition of Protein Denaturation} = \% \text{ Anti-inflammatory Activity}$$

UV visible spectrum

UV visible spectroscopy was performed to estimate the purity of the collagen particles extracted using acid solubilizing method.

FTIR spectrum

FTIR (Fourier Transform Infrared Spectroscopy) was performed to identify and to detect the peak of the active components present and newly formed compounds by bonding and stretching in the collagen, plant extract and collagen infused plant extract.

RESULTS AND DISCUSSION

Estimation of protein: The protein content present in crude collagen was estimated using

Lowry method with some modifications. The presented graph showed the protein content to be 81.5 mg/100 mg of sample. (Abs of sample = 0.035). The result in graph-1 shows higher amount of protein content, higher the protein concentration influenced by the high amount of amino acids content in the collagen. Concur ring to Veeruraj *et al.*,¹⁷ glycine is a major amino acid present in the collagen. This is basic for the arrangement of super helical structures of collagen that makes a difference in tissue. Furthermore, this amino

acid content also has an influence on the stability of collagen fibre, shrinkage and denaturation temperature of the collagen^{18,19}.

Solubility of collagen at different pH

The most elevated dissolvability was watched at pH 1, pH 3 and pH 5 separately (Table-1, Graph-2). Agreeing to Wu *et al.*,²⁰ collagen ought to be more soluble in acidic pH, within the range of pH 1 to 4 and less soluble in isoelectric point. Since within the acidic pH, the charge will be positive which lead to a solid electrostatic repulsion between



Fig. 1. Preparation of aqueous extract of *Cassia auriculata*



Fig. 2. Infusion of collagen with *Cassia auriculata*

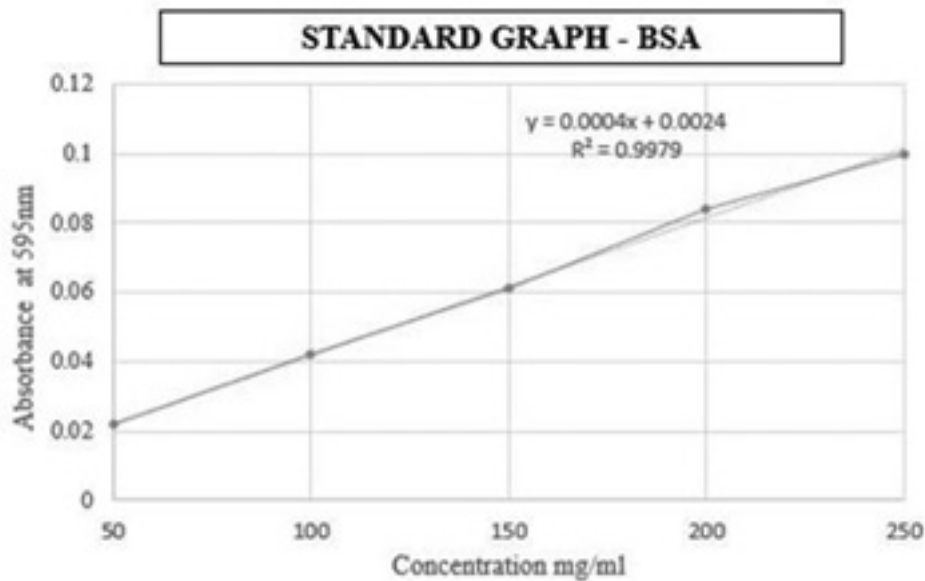
collagen molecules. This study compared with findings of Veeruraj *et al.*¹⁷ higher solubilisation occurred in acidic pH from 1 to 5.

Phytochemical analysis of plant extract

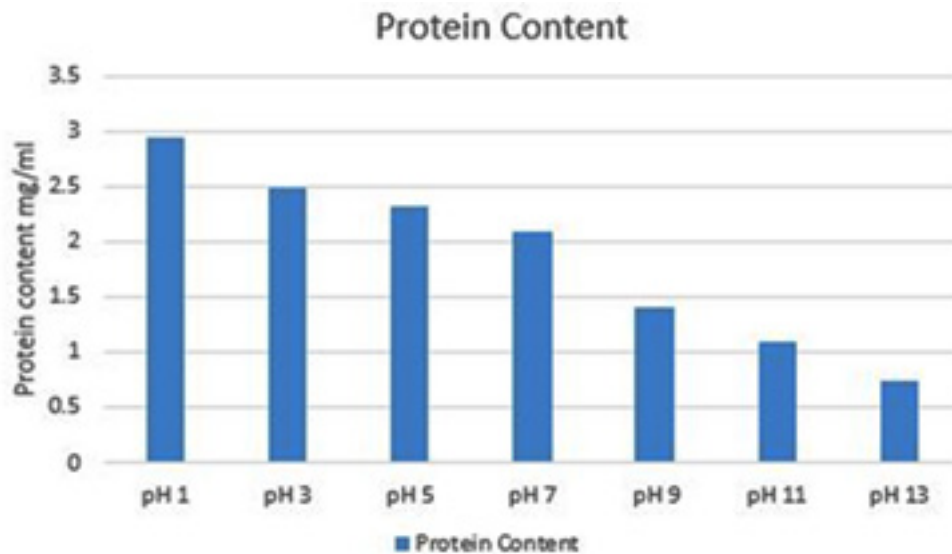
The phytoconstituents were identified by chemical tests, which showed the presence of various phytoconstituents in plant extract of *Cassia auriculata*, reveals the presence of flavonoids, phenols, tannins, terpenoids in maximum amount (Table -2)

Phenols

Phytochemical screening of the aqueous extract revealed the existence of phenolic compound in the extracts (Graph-3). By using Folin Ciocalteu method the total phenolic content was determined in terms of the Gallic acid equivalent (GAE) in mg/g of the extract. With the help of graph, the total phenolic content was determined and the standard curve equation was $y = 0.0004x - 0.0732$, where



Graph 1. Protein content present in crude collagen



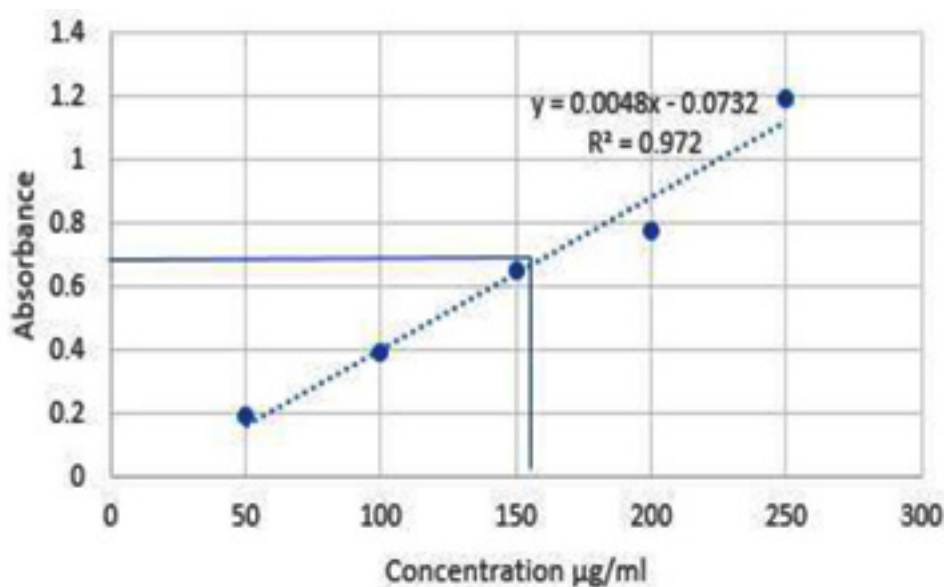
Graph 2. Protein solubility at Different pH

Table 1. Solubility of collagen at different pH

pH	Protein Content mg/ml	Percent of Relative solubility
pH 1	2.94	98
pH 3	2.5	83.33
pH 5	2.32	77.33
pH 7	2.1	70
pH 9	1.4	46.67
pH 11	1.1	36.67
pH 13	0.75	25

Table 2. Phytoconstituents present in plant extracts

S.No	Phytochemical	(Crude extracts)
1	Alkaloids	+
2	Flavonoids	++
3	Saponins	+
4	Tannins	+++
5	Phenol	+++
6	Terpenoids	++
7	Glycosides	+
8	Steroids	-

**Graph 3.** Phenolic compound present in the plant extract

$R^2 = 0.972$. The total phenolic contents (Gallic acid equivalents, mg/g) in the aqueous extracts were calculated to be 150.66 $\mu\text{g/ml}$.

Flavonoids content

Aqueous extract of the plant shows plentiful amount of phenolic group of phytochemicals (Graph-4), one of the most bioactive ingredient present in it. Hence quantitative assessment of flavonoids, a sub class of phenol was also performed for the sample. The total contents of flavonoids were determined by colorimetric method. The solution was mixed well, and the absorbance was measured at 510nm. To measure the standard curve, Quercetin was used ($y=0.0007x+0.054$, R^2

$= 0.9759$) and the results were expressed as 210 μg of Quercetin equivalents (CEs) per g of extract.

Phenols, alkaloids, flavonoids and terpenoids exhibit excellent anti-inflammatory activity, through any of the several cellular mechanisms like, altering the activities of immune cells involved in inflammatory reaction like the macrophages and neutrophil, modulating the proinflammatory gene expression, or by regulating the functional expression enzymes, as mentioned by Kolapo AL *et al.*,²¹.

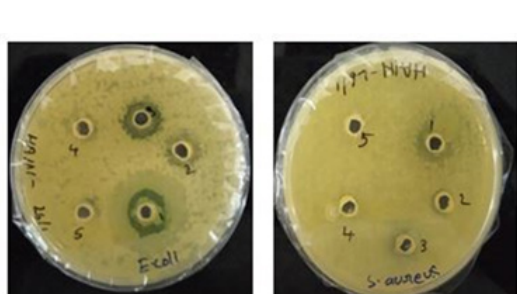
Antibacterial activity

The results of antibacterial activity of plant extract and collagen infused plant extract of

Cassia auriculata against Gram negative bacteria *E. coli* and Gram positive bacteria *S. aureus* are reported in table-3 and 4. The concentration showed higher average zone of inhibition was 100mg/ml as mentioned by Sudharsana *et al*²². Our study results in accordance with Hosamani *et al* 2011 and Scalbert, 1991. In their study observed that antimicrobial activity of *Cassia auriculata* may be

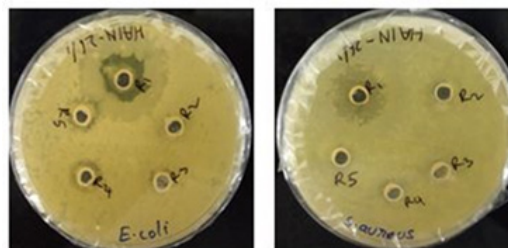
due to the presence of phytochemical constituents like flavonoids and phenolic compounds present in the plant as secondary metabolites. In our study we found that the high amount phenol, Tannins and Flavonoids content^{23,24}.

Plant extract showed greater ZOI towards gram positive bacteria (Plate-1). Whereas collagen infused plant extract showed greater ZOI towards



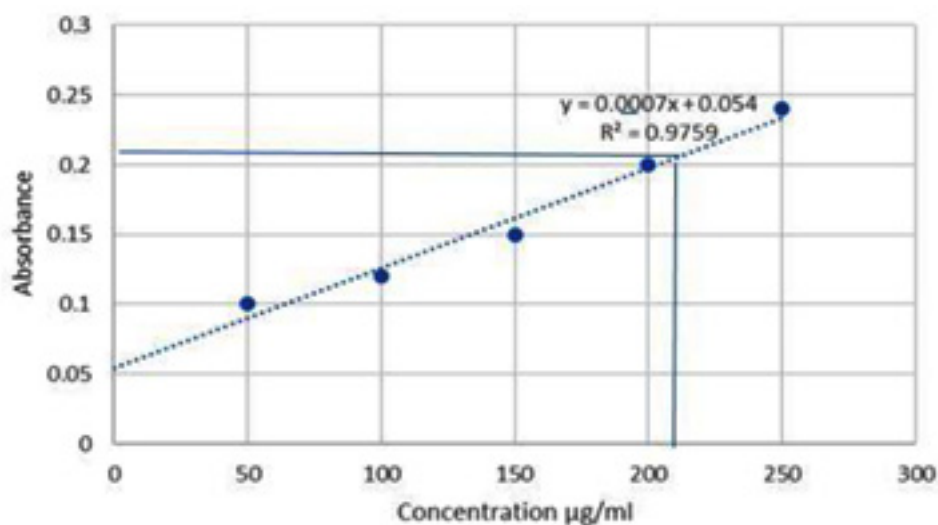
PLANT EXTRACT

Plate 1. Antibacterial activity of plant extracts



PLANT EXTRACT INFUSED WITH COLLAGEN

Plate 2. Antibacterial activity of collagen infused plant extract



Graph 4. Flavonoids content present in the plant extract

Table 3. Antibacterial activity of *Cassia auriculata*

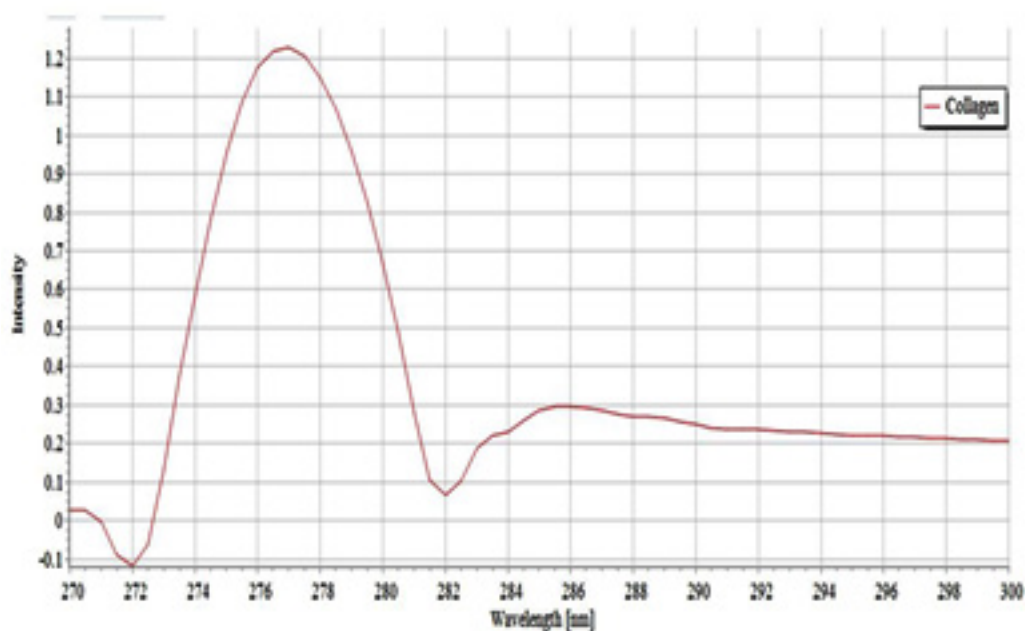
Sample code	Compound	Zone of Inhibition in mm (<i>E.coli</i>)	Zone of Inhibition in mm (<i>S.aureus</i>)
01	Positive standard	22	17
02	Plant extract – 100 mg	15	13
03	Plant extract – 50 mg	14	-
04	Plant extract – 25 mg	-	-
05	Negative control	-	-

Table 4. Collagen infused plant extract against Gram negative (*E. coli*) and Gram positive bacteria (*S. aureus*)

Sample code	Compound	Zone of Inhibition in mm (<i>E.coli</i>)	Zone of Inhibition in mm (<i>S.aureus</i>)
R1	Positive standard	23	19
R2	Plant extract – 100 mg infused collagen	15	13
R3	Plant extract – 50 mg infused collagen	14	12
R4	Plant extract – 25 mg infused collagen	13	11
R5	Negative control	-	-

Table 5. Anti-inflammatory activity of plant extracts and plant extracts infused with collagen

Plant extract	Absorption @660nm	Percentage inhibition (%)	Plant extract infused with collagen	Absorption @660nm	Percentage inhibition (%)
Positive standard	0.412	20.17	Positive standard	0.412	20.17
Plant extract-100mg	0.565	16.27	Plant extract-100mg infused with collagen	0.510	17.25
Plant extract-50mg	0.542	13.00	Plant extract-50 mg infused with collagen	0.595	13.42
Plant extract-25mg	0.654	7.32	Plant extract-25 mg infused with collagen	0.641	9.24
Negative control	0.689	2.18	Negative control	0.689	2.18

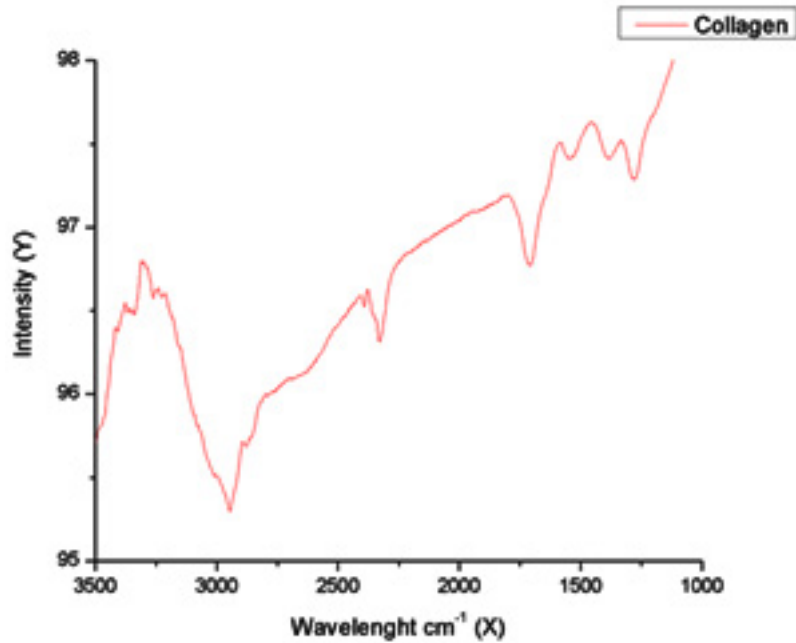
**Graph 5.** Extraction of collagen by acid solubilizing method

both gram positive and gram negative bacteria (Plate-2).

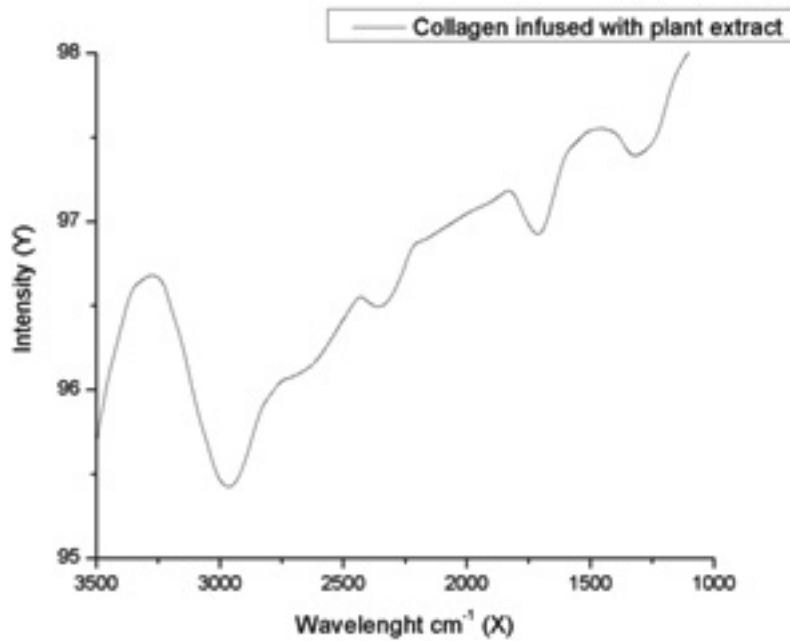
Anti-inflammatory assay

Anti-inflammatory test was done at 660nm, with absorbance values. OD value can

be obtained to calculate inhibition percentage. Here plants extract showed percentage inhibition for 100mg / ml and the collagen infused plant extract showed slightly higher percentage of inhibition for 100mg / ml (Table-5). Our study in



Graph 6. FTIR spectrum of collagen molecule



Graph 7. FTIR spectrum of collagen infused plant extract

accordance with the Lilian *et al*, 1998. They have reported that methanolic extract of *C. auriculata* leaves showed potent anti-inflammatory activity compared to aqueous, hydroalcoholic and ethyl acetate extracts ²⁵. As the anti-inflammatory effect was more significant during later phase of inflammation, it can be concluded that there might be inhibition of inflammatory mediators such as prostaglandins, leukotrienes, polymorphonuclear cells or bradykinins etc., This anti-inflammatory activity due to presence of active phytoconstituents like steroids, flavonoids, alkaloids, terpenoids, tannins and other phytochemicals.

UV visible spectrum

The UV spectrum showed maximum absorbance around 277nm. This defines that the acid solubilizing method was successful in extraction of collagen with high purity of protein content (Graph-5)

FTIR spectrum

The FTIR result in disappearance of the peaks at 1500 cm⁻¹ and 2250 cm⁻¹ on comparing between graph 6 and graph 7 indicates formation of new group thereby bonding between collagen and plant extract. As mentioned by Manuel J. seixas ²⁶ the result showed that some frequency of stretching in the collagen samples which refers to new formation of compounds.

CONCLUSION

In conclusion, the collagen was successfully extracted from the *Cattla Cattla* fish scales using acid solubilizing collagen extraction method and collagen was infused with plant extract of *Cassia auriculata*. The results obtained from this study confirmed that final product prepared using collagen and plant extract can be used to prepare the dressing material such as gels, ointments, bio-films, powdered flakes etc. This combination can help in effective wound healing and can act as great alternative dressing material for those who are having diabetic wound. Since diabetic person have a low potential in wound healing mechanism, this can enhance the wound healing process. It can be used to treat in various stages and types of wound in medical field. In short, the characteristics of the isolated collagens obtained from this study are similar to those of commercial collagen, indicating that a value-added product could be produced from

Cattla Cattla fish. Nevertheless, the use of collagen substitutes to fasten wound healing processes has attracted by many researchers and manufacturers in industry.

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Conflict of interest

The authors declare no conflict of interest.

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