

Research Article



Collagen – *Azadirachta indica* (Neem) Leaves Extract Hybrid Film as a Novel Wound Dressing: *In vitro* Studies

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ABSTRACT

Diabetes is a group of metabolic diseases and it caused the formation of foot ulcers that is difficult to treat and occasionally require amputation. India has a number of diabetic patients and it is increasing nowadays. Impaired wound healing is an important clinical problem in diabetes mellitus which causes failure in healing of diabetic patients. In this study collagen was used as a wound dressing material with neem leaves extract impregnated in it. Neem leaves extract is used traditionally in therapy of skin disorders. Collagen was isolated from chrome shavings and impregnated with neem extract. Ethylene glycol (EG) was used as a crosslinker. Physical characterisation was done using SEM, FTIR, tensile strength, water absorption studies, and porosity measurement. Biocompatibility test were done using RAW 264.7 cell lines and amount of nitric oxide scavenged was determined using Griess reagent. Antioxidant assays were performed using DPPH, hydroxyl radical scavenging, reducing power. Physical characterisation results of the biomaterial showed more than 100% water absorption capacity and porosity greater than 50%. DPPH assay showed an optimum scavenging of 80% for collagen sheets with 400µg/ml of neem extract in it. The prepared bio composites were found to have more than 80% cell viability.

Keywords: Collagen, antioxidant, neem leaves, RAW 264.7, impaired wound healing, ethylene glycol.

INTRODUCTION

Wound healing is a complex process composed of several overlapping phases containing hemostasis, inflammation, proliferation and remodelling¹. Due to micro and macro-vascular complications impairment in wound healing occurs especially in diabetic patients.

Co-ordination of various cells, cytokines and growth factors are required for wound healing process. Initial phase of wound healing is inflammation, where macrophages mainly take the role. They are responsible for removing non-functional host cells and bacteria. Besides, they co-ordinate and repair through production of a broad spectrum of factors that influence angiogenesis, fibroplasias and extra cellular matrix synthesis². Delay in wound healing process occurs due to increase in oxidative stress caused by over production of free radicals³. Introduction of an antioxidant to eliminate the free radicals can help improve diabetic wound healing⁴.

Wound dressings made from natural body components may be advantageous in order to improve biocompatibility and to avoid inflammation or rejection. Collagen is present in the extracellular matrix (ECM) and acts as a natural substrate for cellular attachment, growth and differentiation^{5,6}. The main objective of using wound dressing is to accelerate wound healing by preventing bacterial infection and the quickening of tissue regeneration. Wound dressing materials should possess essential properties like flexibility, durability, gas permeability and ability to prevent water loss. Collagen as base material offers several advantages, which has well-

documented structural, physical and chemical properties. Moreover, collagen has low antigenicity, low inflammatory, good biocompatibility and has the ability to promote cell attachment and proliferation^{7,8} and also supports the formation of new granulation tissue and epithelium at the wound site (Bertone). Collagen is largely employed as dressings for wound repair due to their biodegradability, biocompatibility and low immune reactivity (Parenteau-Bareil). Collagen alone cannot assist the healing of infected wounds because it is protein in nature and bacteria can use collagen as a substrate. Because of the imbalance between host resistance and bacterial growth, infection of the wound occurs. Plant extracts possessing active ingredients are incorporated into biomaterials for better performance against bacterial infection. *Azadirachta indica* is a traditional medicinal plant in India. In India, for curing wounds, cuts and other skin diseases *Azadirachta indica* have been widely used by various tribes⁹. Medicinal properties of its leaves such as antioxidant and antimicrobial activity were contributed by the phytoconstituents present in them. The lavenoids present in them act as antioxidants which provide protection against free radicals that damage cells and tissues. The lavenoids present in them act as antioxidants which provide protection against free radicals that damage cells and tissues and also the tannins promotes wound healing (Majewska).

Chrome containing leather wastes (CCLW) are the prominent solid wastes in tanning industry. Chromium is toxic and it makes the disposal of CCLW a serious problem from environmental point of view. The CCLW mainly consists of collagen and Cr(III) complexes, which could be treated to give the potential resources of



collagen protein and chromium^{10,11}. In the present study, the wound dressing material is prepared using collagen isolated from chrome shavings and impregnated with neem leaves extract using ethylene glycol as a cross linker and it is evaluated for its physical characteristics and antioxidant potential of wound dressing material. *In vitro* characterization of biomaterials is both ethically and economically critical as it greatly reduces the number of animals necessary for the *in vivo* tests. *In vitro* studies are crucial for evaluation and prediction of the corrosion, biodegradation, solubility, bioactivity, and biocompatibility of biomaterials in physiological environment.

MATERIALS AND METHODS

Materials

CCLW was obtained from local leather tanning industry. Cell lines (RAW 264.7) were purchased from NCCS, Pune. DPPH, potassium hexacyanoferrate, 1,10-phenanthroline, sulphanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride were purchased from HiMedia. All the chemicals used were analytical grade and used without any further purification.

Isolation of Collagen

The CCLW was dechromed by treating it with 99% concentrated sulphuric acid. After the treatment, it was washed using distilled water for 5-6 times.

The demineralised chrome shavings were then treated with hydrogen peroxide for bleaching. Resulting collagen solution was lyophilised and stored for further analyses.

Preparation of Extract

Leaves of *Azadirachta indica* were collected and shade dried for 10 days. Dried leaves were ground to fine powder using mixer grinder. 20 g powdered neem leaves were subjected to extraction using methanol for 24 h.

The extract was concentrated in rotary evaporator and lyophilised. It was further stored at 4°C until use.

Preparation of Bio Composite

Collagen solution was prepared using 0.05M acetic acid in the ratio 1:10. The solution was stirred 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 extract was added to 20ml of collagen solution and mixed well.

Varying amounts of ethylene glycol was added (0.5ml-2.5ml). Solution was poured in plates and air dried¹². The prepared collagen sheets were stored for further analysis.

Characterization

Morphological study

Surface morphology of the bio composite was visualised by scanning electron microscope. Gold coating was done on bio composites using ion coater 0.1Torr pressure,

20mA current, and 70s coating time, using a 15kV accelerating voltage.

FTIR

Fourier transform infrared spectroscopy was done to determine the functional groups in collagen bio composites.

The spectra was measured in the frequency range of 4000-500 cm⁻¹ using Nicolet 360 Fourier Transform Infrared (FTIR) spectroscope.

Water Absorption Studies

The water absorption capacity of bio composites prepared was determined by swelling small pieces of each bio composite of known weight in distilled water at room temperature¹³. Weight of bio composites were noted after blotting it with filter paper to remove excess water on its surface and was recorded every 1hr, 2hr, 3hr and after 24hr. Percentage water absorption of the bio composites at a given time was calculated from the formula:

$$E_s = \frac{(W_s - W_o)}{W_o} * 100$$

Where W_s - weight of the bio composite (moist) at given time, W_o - initial weight of the bio composite. E_s is the percentage of water absorption at a given time. The results given are average of three bio composites.

Porosity Measurement

Bio composite porosity and density were determined via liquid displacement method using ethanol as the displacement liquid because of its easy penetration through the pores of the bio composites and which will not induce shrinking or swelling as a non solvent of the polymers¹⁴.

A known weight (W) of the bio composite was immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The bio composites were kept in ethanol for 5 min, and then a series of brief evacuation-repressurization cycles were conducted to force the ethanol into the pores of the bio composite.

The process was repeated until the air bubble stops. The total volume of ethanol and the ethanol impregnated bio composites was then recorded as V_2 .

The difference in the volume was calculated by ($V_2 - V_1$). The bio composite impregnated with ethanol was removed from cylinder, and V_3 is the residual ethanol volume. Thus, total volume of the bio composite was $V = (V_2 - V_1) + (V_1 - V_3)$

Porosity of the bio composite was obtained with, ϵ

$$\epsilon = \frac{V_1 - V_3}{V_2 - V_3}$$

Tensile Strength

Bio composites of 15mm width and 5cm length were obtained from the prepared bio composite.



Mechanical properties such as tensile strength (MPa) and percentage of elongation at break (%) were measured using a universal testing machine. Results were given in average of three specimens.

Antioxidant Activity Measurements

DPPH Radical Scavenging Activity

Various concentration of bio composites were added to 0.1mM 2,2-diphenyl-1-picrylhydrazyl(DPPH) in methanol. The reaction mixture was mixed thoroughly and incubated for 30 min at 37°C in dark. Absorbance was measured at 517nm¹⁵. Percentage radical scavenging activity was calculated using the formula:

$$\% \text{ Radical Scavenging} = \frac{OD \text{ of DPPH alone} - OD \text{ of test sample}}{OD \text{ of DPPH alone}}$$

Hydroxyl Radical Scavenging Activity (HRSA)

Hydroxyl radical scavenging of collagen and collagen with neem was done according to Li¹⁶. Fenton reaction was used to produce hydroxyl radicals. Hydroxyl radicals can convert Fe²⁺ into Fe³⁺. 1,10-phenanthroline can only combine with Fe²⁺ to form red coloured compound with an absorbance at 536nm. 1ml 1,10-phenanthroline solution (1.5mM) and 1.0ml of bio composite was added in order and mixed well. 1.0ml of FeSO₄.7H₂O is added to the solution and 0.03% 1.0ml H₂O₂ is added to initiate the reaction. Solution mixture was then incubated for 1h and absorbance was measured at 536nm. Negative Control was the reaction mixture without antioxidant (An), reaction mixture without hydrogen peroxide was kept as blank (Ab).

$$\text{HRSA (\%)} = \frac{(As - An)}{Ab - An} \times 100$$

Reducing Power

Bio composites were dissolved in water and mixed with 1% potassium hexacyanoferrate and incubated for 30 min at 50°C. After incubation 10% trichloroacetic acid was added to the reaction mixture and mixed well. 2 ml of supernatant from the resultant reaction mixture was then mixed with distilled water and 0.5ml of ferric chloride was added to it. Absorbance was measured at 700nm¹⁷. Increase in absorbance indicates increase in reducing power.

Cytotoxicity Studies

RAW 264.7 macrophage cells were cultured in DMEM medium supplemented with 10% FBS and penicillin/streptomycin (10U/ml) at 37°C in 5% CO₂ incubator. Once the cells reached 70-80% confluency, the cells were trypsinized using 1X tyrsin-EDTA and passaged. When the cells reached at least 80–90% confluency, they were trypsinized for passaging. Collagen biosheets were cut in 10mm diameter and kept in 24 well plate and UV sterilized for 30 min. Bio composites were preincubated with medium without serum overnight. 1*10⁵ cells were seeded onto each well. Cells alone group were kept as control. Triton X added wells were the positive control.

After attaining 80% confluency MTT (5 mg/ml) was added onto each well and incubated in dark for 4 hrs. After incubation, DMSO (500µl) was added in each well. Absorbance was measured at 540nm¹⁸.

RESULTS AND DISCUSSION

Collagen is a natural component present in skin and very essential for wound healing. Collagen isolated from chrome shavings is a value added product which can reduce the cost of collagen used for bio composite preparation and it also helps in reducing waste from tannery industry in the form of chrome containing leather waste¹⁹. Neem leaves are traditionally used medicine to cure cuts and wounds. It also has the advantage of natural compound which helps reduce any rejection due to foreign materials. It also has significant antioxidant and antimicrobial activity which contributes to its wound healing effects⁸. Collagen is a good carrier for drug release. Plant extract incorporated into collagen sheets thus used for wound healing can have more efficiency due to drug release from collagen sheet will be more specific.

Collagen bio composites were made using different concentration of neem extract in it using varying concentration of crosslinker. If a material is brittle or its mechanical properties diminishes when comes in contact with water or wound exudates, it cannot be used as a wound dressing material. Ethylene glycol was used as a cross linker and the mechanical properties of the material is found to be increased and is made suitable for biomedical applications. Mechanical property evaluation was done using tensile strength measurement and it was maximum for 0.5ml of EG containing bio composites.

Water Absorption Studies

Maintaining a dry wound surface is required for enhanced wound healing and hence the material should possess good water absorption capacity and it should also retain its shape intact. The bio composites prepared were evaluated for water absorption capacities (Figure 1) and the results shows more than 100% water absorption for all the bio composites which makes its suitable to be applied for wounds and all the bio composites retained its shape till the end of testing.

Porosity Measurement

Pore size, surface area and porosity of bio composites are essential for cell attachment and migration which are important parameters in wound healing. Porosity of the material is evaluated using liquid displacement method and a porosity of 50% was found for all the materials. Porosity is considered as an important parameter in all tissue engineering applications^{20,21}. Porosity and density measurement result showed that porosity decreases with increase in neem extract content in it. On the other hand density of the sheets showed an increase in density with increase in neem extract used. Porosity is an important criteria for selection of bio composites. Collagen with



neem impregnated sheets showed highest porosity in sheets with 0.5ml (Figure 1) and 1.0ml ethylene glycol

(result not shown). Other bio composites showed similar porosity with increase in ethylene glycol in it.

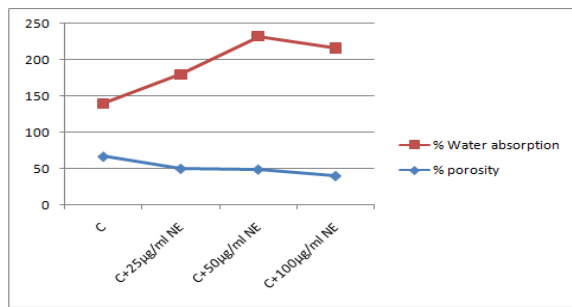


Figure 1: Percentage water absorption and porosity of collagen biocomposites prepared with 0.5ml of EG. C- collagen.

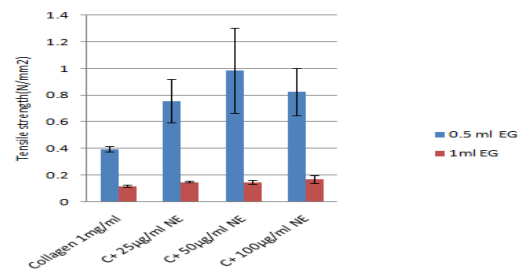


Figure 2: Tensile strength of collagen bio composites with varying concentrations of neem extract made with 0.5ml and 1.0ml of EG as crosslinker.

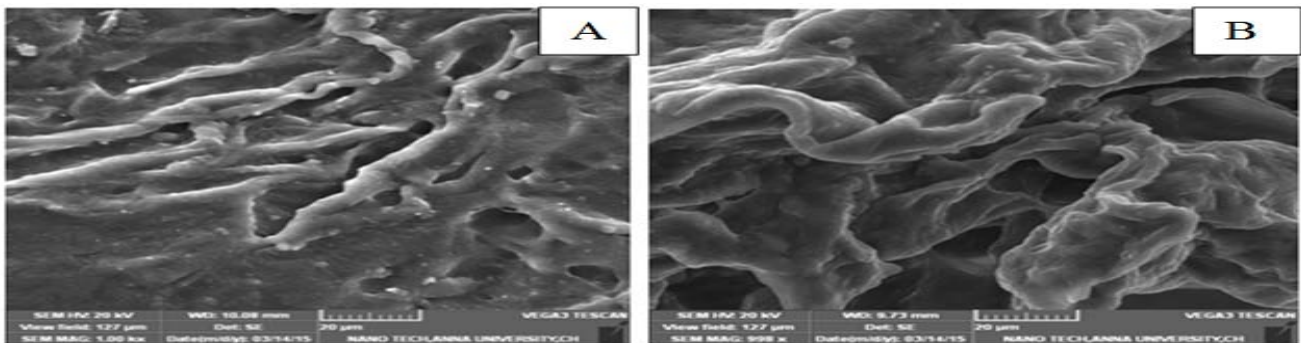


Figure 3: A- SEM image of collagen sheet, B-SEM image of collagen with neem extract (100µg/ml)

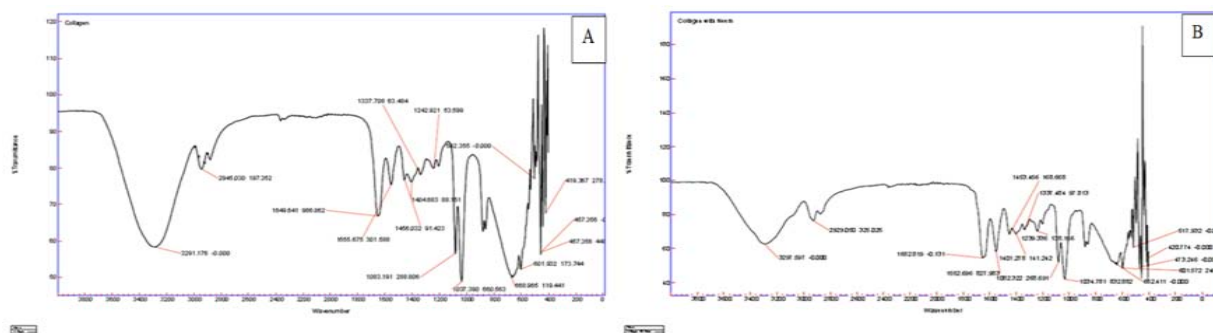


Figure 4: A-FTIR spectra of collagen isolated from chrome shavings, B-FTIR spectra of collagen impregnated with neem extract

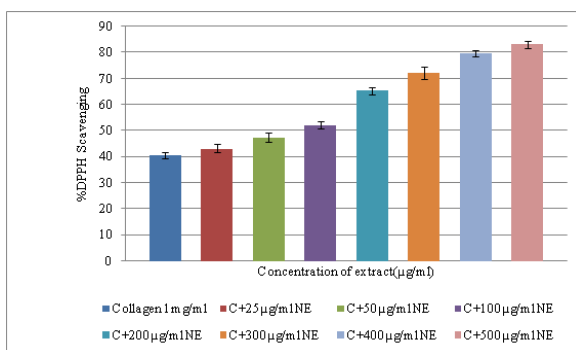


Figure 5: Scavenging effects of collagen and collagen with different concentrations of neem extract on DPPH radicals. Radical scavenging was measured by absorbance at 517nm.

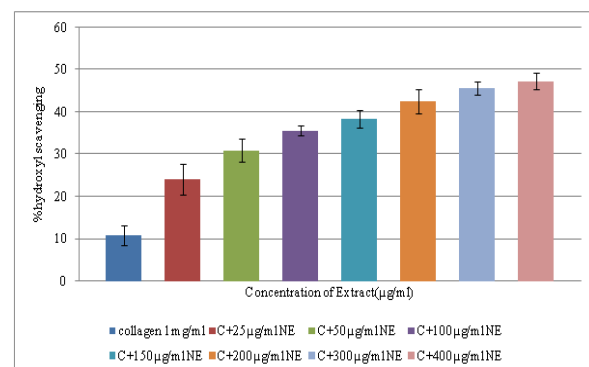


Figure 6: Dose dependent hydroxyl radical scavenging of collagen and collagen with different concentrations of neem extract measured at 540nm.

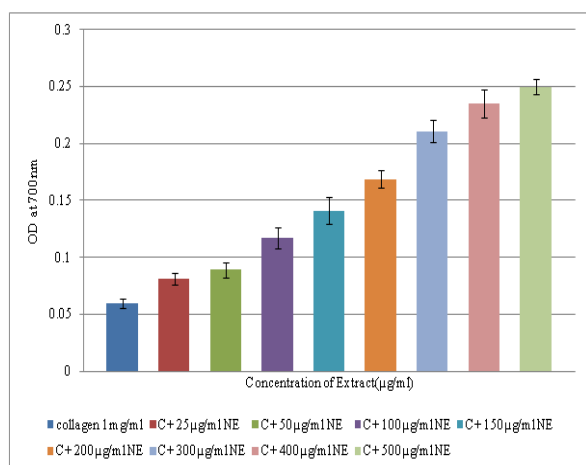


Figure 7: Reducing power (absorbance at 700nm) of collagen and collagen with neem extract of different concentration.

Tensile Strength

Figure no 2 illustrates the tensile strength of collagen and collagen with neem sheets prepared with two different concentrations of ethylene glycol. As the volume of EG increased the tensile strength decreased. With increase in concentration of neem extract in collagen sheet doesn't show any significant difference in tensile strength. More than 1 MPa is possessed by all the bio composites prepares with low volume of EG in it. Tensile strength is an important parameter for a wound dressing material to ensure easy handling. Percentage elongation is also found to be moderate for low concentrations of crosslinker used which is also suitable for the wound dressing material.

SEM and FTIR Analysis

SEM analysis was done and it showed the presence of pores in the material (Figure 3A). Incorporation of plant extract into the collagen sheet made it more smooth and images obtained showed a fibrous structure (Figure 3B).

Collagen has 3 characteristic amide peaks and its presence can be used to confirm the presence of collagen in the prepared sheets. Amide peaks at 1649cm^{-1} , 1555cm^{-1} , and 1242cm^{-1} were obtained in the FTIR results of composites made of collagen (Figure 4A). A slight shift in peaks was obtained for sheets with plant extract in it showed the successful incorporation of plant extract in the collagen sheets (Figure 4B).

Antioxidant Activity

DPPH radical scavenging assay is one of the quick methods for evaluation of antioxidant activity. DPPH is a free radical with relatively stable nitrogen centre which can accept a hydrogen radical or electron to become stable²². Di phenyl picryl hydrazyl gets converted to hydrazine upon accepting electron. DPPH is violet coloured solution which turns into yellow solution upon addition of reducing agent and can be measured using spectrophotometer at 517nm, increase in antioxidant potential is indicated by decrease in OD with respect to

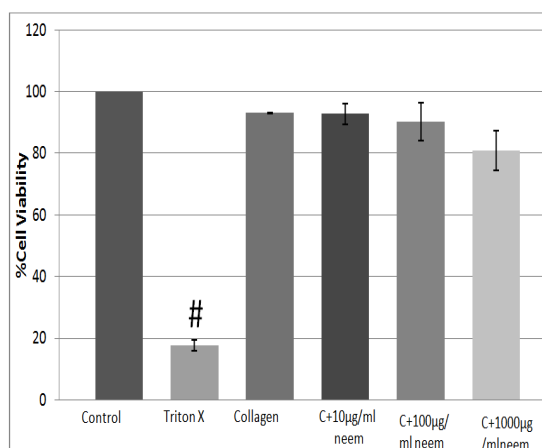


Figure 8: MTT results for collagen sheets impregnated with neem extract. Control viability was set as 100%. Results were given as mean \pm SD (# $p < 0.01$).

control²³. In the assay, collagen and collagen with varying concentrations of plant extract were evaluated for their free radical scavenging activity.

The scavenging effect increased with the increasing concentrations of neem in collagen. From the results of DPPH, It showed that collagen itself possess free radical scavenging activity upto 40% and it increases to more than 80% with increase in concentration of neem extract (Figure 5).

Hydroxyl radical scavenging and reducing power assays were also done to check antioxidant potential. Hydroxyl radical scavenging showed a maximum of 45% scavenging and further increase in neem concentration doesn't show any significant increase in scavenging effect (Figure 6).

Reducing power analysis showed an increase in absorbance with increase in concentration neem extract in collagen. This shows increase in antioxidant activity with increase in neem extract concentration with collagen (Figure 7).

Oxidative stress can cause various diseases and conditions like impaired wound healing, prolonged inflammation, diabetes etc. Wound dressing material with antioxidant potential can enhance proliferation²⁴ and helps prevent apoptosis²⁵. Collagen is a widely used biomaterial as wound dressing material which possess antioxidant potential²⁶.

From the current study it is revealed that with addition of neem extract antioxidant potential has been enhanced. Thus the use of collagen with neem can reduce cell damage occurred due to oxidative stress efficiently.

Neem extract contains flavanoids, alkaloids, steroids, saponins and tannins⁸.

Antioxidant potential is due to the presence of flavanoids²⁷, thus preventing cell damage due to free radicals and wound healing property is due to tannins. Saponins has anti bacterial²⁸, antifungal²⁹ and antiprotozoal³⁰ potential.

Biocompatibility

For biomedical applications the wound dressing material should be biocompatible. Biocompatibility of collagen with neem biosheets were done using MTT assay. This assay is used to determine presence of metabolically active cells spectrophotometrically.

Tetrazolium dye is converted to formazan by NADPH dependent oxidoreductase enzyme³¹. Formazan crystals are formed inside the cells, thus they are solubilised using DMSO and spectrophotometric analysis is done at 540nm. Control comprised of cells alone and obtained results of collagen biosheets were compared with the control. From the results obtained it can be inferred that with respect to control the test material doesn't show any significant decrease in cell viability (Figure 8). This confirms the material is not cytotoxic to cells and can be used for further animal studies.

CONCLUSION

Wound dressing material prepared using collagen isolated from chrome shavings impregnated with neem extract possess both physical and biological properties required for an ideal dressing material.

Presence of antioxidant makes collagen sheet with neem extract can be used for diabetic wound healing and be considered for animal studies since *in vitro* studies doesn't show any significant toxicity for the material.

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