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Evaluation of wound healing and antimicrobial physiognomies of alcoholic extract of *Ocimum sanctum* (basil) in diabetic rabbit model

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Abstract

Diabetes is a chronic metabolic syndrome characterized by impaired wound healing and secondary wound infections attributing to the economy. The plant *Ocimum sanctum* (basil) is widely used owing to its chemo-immuno modulatory, antimicrobial and anti-inflammatory characteristics. Study aims to elucidate therapeutic potential of alcoholic extract of basil in wound healing in diabetic rabbit model. Animals were divided into wounded control treated with povidone Iodine and test with basil extract. Semi-quantitative and qualitative evaluation of tissue architecture and immuno-histochemistry encompassing collagen I and III studies expressed swift regeneration of injured epidermis in basil extract treated wound in comparison to control. This is articulated by organized dermal collagen deposition with collagen I to III ratio resembling normal skin, wound area measurement and absence of clinical signs of wound infection. Encouraging primary evidences for swifter wound healing requisites further experimental validation for the translation and standardization of the product from bench to bedside.

Keywords: Diabetes Mellitus, *Ocimum sanctum*, wound healing, antimicrobial activity

1. Introduction

Diabetes mellitus (DM) is one of the most rampant chronic diseases, estimated to reach up to 439 million by 2030 and chronic diabetic wounds attributing to the morbidity is an important public health issue [1]. Diabetics are predicted to have 25% more lifetime risk of developing a foot and hundred times greater hazard of a lower extremity amputation in comparison to the euglycemic counterparts [2]. Chronic wounds resulting from neurovascular complications of the same display anomalous healing, characterized by persistent inflammation and high infection rate [3, 4].

Collagen, constituting 30% of the total protein in the human body is an innate substrate for cell attachment and differentiation and accurate arrangement of collagen fibrils into systematic assemblies is vital for restoring anatomic structure and function following tissue injury [5]. Fibrils within the extracellular matrix exhibit distinctive banded pattern with a periodicity of 67 nm known as D-banding. The fine-woven fibrillar collagen in the papillary dermis can be evidenced from the coarse collagen bundles of the reticular layer [6]. Balanced deposition of collagen I and III is also obligatory as small deviance in the matrix composition during wound repair can ominously modify the final disposition of the healed wound [7]. Diabetic patients have displayed to have impaired wound healing owing to associated with decreased amounts of collagen fibrils. Post-translational modifications of the collagen peptide in diabetes primes to accelerated cross-linking of skin collagen with decreased solubility alongside augmented accumulation of collagen in dermal tissues owing to increased half-life [8]. Advanced Glycation End products reaped up from hyperglycemia ensuing in non-periodic collagen fibrils have poor tensile strength which get ruptured under cellular pulling forces [5, 9].

Fibroblasts sequestered from diabetic ulcers displayed senescence and a diminished proliferative response to growth factors like TGFβ1, platelet-derived growth factor alongside other cytokines, with compromised phosphorylation of transduction signals, comprising Smad2, Smad3, and mitogen-activated protein kinase [10, 11]. Consequently, during the course of diabetes, the customary mechanical strength provided by collagen to wound bed gets deformed owing to physicochemical changes such as additional glycation, shortfall of docility and resistance to enzymatic digestion by being transformed to a plastic from an elastic matrix [12-14]. Though more work is necessary to clearly define the phenotypical aberrations in diabetic wound cells and collagen production anomalies, these findings have precise inferences for therapeutic intercession.

Currently in comparison to synthetic drugs, traditional and herbal medicine is gaining popularity due to its widespread availability, moderate efficacy, acceptancy, no or fewer side effects and low cost especially in developing economies. Many of the currently used novel treatment regime like recombinant growth factors or tissue-engineered wound dressings are very expensive and not available for many patients in the developing countries. Moreover chronic wound management exerts substantial financial burden to the health-care system [15].

Charaka Samhita by Charaka, an ayurvedic text suggest numerous applications of *Ocimum sanctum* (also known as Tulsi, *O. sanctum*, basil) including treatment of parasitic infections, gut disorders, skin & joint diseases, ophthalmic conditions and so on. Its extracts have found application in common colds, headaches, stomach disorders, inflammation, heart disease, various forms of poisoning and malaria. Earlier studies have shown pronounced hypoglycemic effect of *O. sanctum* extract on oral consumption in diabetic rodent model. Based on previous animal studies *O. sanctum* may act at several levels in the immune mechanism involving antibody manufacture, hypersensitivity reaction intermediaries release and the humoral immune responses modulator [16, 17]. Thus it suggests that diverse mechanisms like free radical foraging, metal chelation alongside immune modulation may operate at different echelons individually or in amalgamation to bring about the wound healing effects. Significant inhibition of growth of pathogenic microorganisms was observed in vitro by traditional drugs like *O. sanctum*, Scientific validation of its effectiveness in diabetic wound healing could result in development & standardization of novel products to address the grave concern and ensure its safe use [18]. Appreciating the tremendous pharmacological potential and the wealth of available literature, current study highlights the role topical application of *O. sanctum* extract in diabetic wound healing and its potential as an adjunct therapy for chronic wounds.

2. Materials and Method

2.1 Extract preparation



Fig 1: Image of the selected plant *Ocimum Sanctum* (basil)

The stem leaves and flowers of sweet *O. sanctum* were collected from the garden of Indian Institute of Technology Kharagpur. The leaves were identified and verified by botanist Prof. Satyahari Dey, Department of Biotechnology, Indian Institute of Technology Kharagpur (fig.1). Obtained parts of plant were washed with distilled water and dried in the shade at room temperature.

Well-powdered dried plant parts (10g) were filled in the thimble of Soxhlet apparatus for methanol extraction. Dark green colored extract was obtained on reflux condensation for 24 h which was further subjected to rotary vacuum evaporator for concentration. Extract with dichloromethane (DCM) and hexane was evaporated to dried powder to be further dissolved in dimethyl sulphoxide (DMSO). The yield percentage for methanol, DCM and hexane extract was found to be 1.13 g (11.3%), 0.43 g (4.3%) and 0.427 g (4.27%) respectively. The extracts were stored under argon in air tight glass container at low temperature (-20°C).

2.2 Animal model preparation

About 8-10 weeks old (1.88 ± 0.24 kg), New Zealand white male rabbits, 10 in number, of either sex were used for animal model preparation as per the Institute Ethical Committee guidelines (SRGI/COP/SAEC/AM-IITYKGP/16/01).

Animals were kept under controlled environment with alternate light dark cycle and free access to food and water. Animals were acclimatized for 7 days before the start of experiment. For Diabetes induction, light anesthesia was achieved by intramuscular injection of ketamine and xylazine (30 mg/kg and 3 mg/kg respectively) in caudal thigh of well-fed animals, before slow injection of 5% alloxan monohydrate (100mg/kg) in marginal ear vein. Rabbits were provided with food and water immediately on recovery from anesthesia. Each animal was also administered 8 ml of 5% glucose solution subcutaneously at an interval of 4 hours for 12 hours to prevent hypoglycemia episode. Blood glucose was measured every day and subcutaneous regular insulin dose was titred for each animal to keep blood glucose levels (BGL) between 350-400 mg/dl. The animals with target BGL were enrolled for further studies.

Animals were anaesthetized with ketamine/ xylazine and positioned in prone position for surgical wound creation on the dorsum. Full thickness wounds (5 mm depth) were made by stainless steel punch on each animal followed by their random allocation into 3 groups: untreated, *O. sanctum* extract treated and Povidone-Iodine treated [4].

2.3 Biochemistry Analysis

The plasma isolated by centrifugation from blood samples at specific time points was subjected to Blood Urea Nitrogen (BUN), total protein and creatinine testing to identify any laboratory sign of renal failure in the animals.

2.4 Wound swab culture and wound area measurement

Wound swab culture followed by wound site measurement was carried out at specific time points (Day 0, 6, 12) to evaluate rate of healing in all the 2 groups. After digital image acquisition, Image J software was used for precise measurement of wounds. Measurements were triplicated for each animal and mean was calculated.

2.5 Tissue architecture studies and Immunohistochemistry

Tissue samples were collected from the wound margins at specific time points from test as well as control. The samples were processed (formalin fixed, paraffin embedded) and $5\mu\text{m}$ thin sections are mounted on glass slides for further studies.

2.6 Hematoxylin & Eosin (H&E) and Van Gieson's staining

H&E staining was done after deparaffinization to study tissue architecture in healing wound and evaluate the difference in test and control group at specific time points. Van Gieson's

staining was done to study the distribution of collagen and other connective tissue in healing wound tissue samples in test and control [19]. Image acquisition was carried out using Zeiss Observer Z1 Microscope (Carl Zeiss, Germany).

2.7 Immunohistochemistry (IHC)

Tissue sections were baked for 30 minutes at 60°C followed by deparaffinization and gradual alcohol hydration for IHC staining and then subjected to antigen retrieval (EZ-Retriever System V.2; Bio Genex, USA) in 10m Mtris-ethylenediaminetetraacetic acid buffer (pH 9.0). Primary antibody for collagen I and collagen III (Abcam, UK) with dilution 1: 500 were used. Immuno-detection was carried out with Horseradish Peroxidase conjugated secondary antibody with chromogen 3, 3'-diaminobenzidine (DAB) and counterstained with hematoxylin using Super Sensitive Polymer-HRP IHC Detection System kit (Bio Genex, CA, USA).

2.8 Semi-quantitative evaluation of histological and immuno-histochemical features

20 different images of each group were explored independently and one representative image has been modeled and following interpretation was performed with the help of expert histopathologists.

2.8.1 Epithelium thickness measurement:

From the H&E images, the thickness of the epithelium layer was measured vertically from connective tissue interface towards the outermost epithelial lining. Thicknesses (μm) at 10 different random points were measured and their mean was represented with the help of the software Axio Vision Rel. 4.7.2 (Carl Zeiss, Germany).

2.8.2 Assessment of VG intensity variation:

The VG intensity was determined based on a visual perceptual scale on the basis of an intensity scoring scale (i.e., 0–10) at three equidistant points (L1, L2, and L3) within 200 μm range below basement membrane. All the evaluations were carried out by the Axio Vision (version 4.7.2, Carl Zeiss, Germany) software.

2.8.3 Evaluation of collagen I and III expression:

Sub-epithelial staining zones of IHC images were used to study collagen I and III expression. The zones were visually identified using the threshold feature of the Image J software. After thresholding, the colour de-convolution technique was used to un-mix the pure DAB stained areas leaving a complimentary image. The pixel intensities of separated DAB images range from 0 to 300. The darkest shades of the color were depicted by value 0 while 300 displayed the lightest shade of the color in the image. Automated score was assigned by observing and measuring the pure DAB staining pattern and histogram profile of every image was plotted. The histogram profile holds the number of pixels of a specific intensity value against their respective intensity. Therefore, depending on the intensity of color score the collagen I and III expressions were quantified. Ratio between Collagen I and III was calculated from mean values of their intensities.

2.9 Scanning Electron Microscopy (SEM)

Tissue sections of different study groups were observed under SEM (Zeiss Merlin Gemini II) under an accelerating voltage of 10–20 kV to unveil the collagen assembly. Prior to SEM observation, the dried samples were processed and coated

with a thin layer of gold using plasma sputter. Collagen fibril diameter and D spacing was calculated from SEM micrographs using image J software. 20 random fibers were examined for this measurement.

2.10 Statistical analysis

The significance of the epidermal thickness and the collagen intensity was evaluated using Analysis of Variance (ANOVA). The immunohistochemical findings on collagen I and III expressions were also subjected to ANOVA for finding the statistical significance. All the above evaluations were done using SPSS V.17. Furthermore box plots were drawn using MATLABR 2015 b software (Math Works, Natick, MA).

3. Results and Discussion

3.1 Biochemistry evaluation

Nephrotoxicity following the administration of the Alloxan monohydrate in the form of transient azotemia, abnormalities of tubular function, alongside squamous metaplasia was assessed using BUN, total protein and serum creatinine. The BUN concentration was 24.40 ± 1.56 mg /dl, 31.8 ± 1.93 mg /dl and 38.7 ± 1.26 mg/dl respectively in 0,5 and 10 days following the administration of the Alloxan. Serum creatinine level augmented from 0.26 ± 0.12 mg /dl to 1.94 ± 0.25 mg /dl by the end of the study. Total protein remained relatively unchanged with values 5.6 ± 1.12 g/dl and 5.56 ± 1.4 mg /dl in 0 and 10days respectively. The concerned increase in BUN and creatinine can be explicated by an inadequate improvement in the regenerative process in the kidney. Increased serum levels of BUN and Cr in diabetic rabbits could be caused by either renal or non-renal related factors (prerenal azotemia) like stress, severe dehydration, and toxic insults as the rabbit has a limited capacity to concentrate urea [20]. Correlation of the elevation of BUN and Cr to a diabetic nephropathy is difficult, since no histopathological examinations have been performed in the present study.

3.2 General clinical evaluation, Wound swab culture and wound area measurement

Rabbits treated with *O. sanctum* extracts were healthy with no evident symptoms of acute inflammation like rubor, calor and tumor on clinical examination. The formation of the granulation tissue and re-epithelialization was perceived within 4–6 and 6–10 days, correspondingly, following the intervention. As shown in Fig. 2, gross images of wound site at day 6 and 12 shows better healing in *O. sanctum* extract treated wounds compared to PI treated ones. Culture done on swab taken from wound on day 6 and day 12 precluded the presence of pathogenic organism. Bactericidal action is supposed to be due to the presence of Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), linalool and methyl chavicol [21]. The total wound area (pixel value) for *O. sanctum* treated wound as calculated by Image J software was found to be significantly reduced when compared to control ($p > .01$). *O. sanctum* extract treated wound shows swifter healing rate when compared to the PI one where epithelization was completed by day 12.

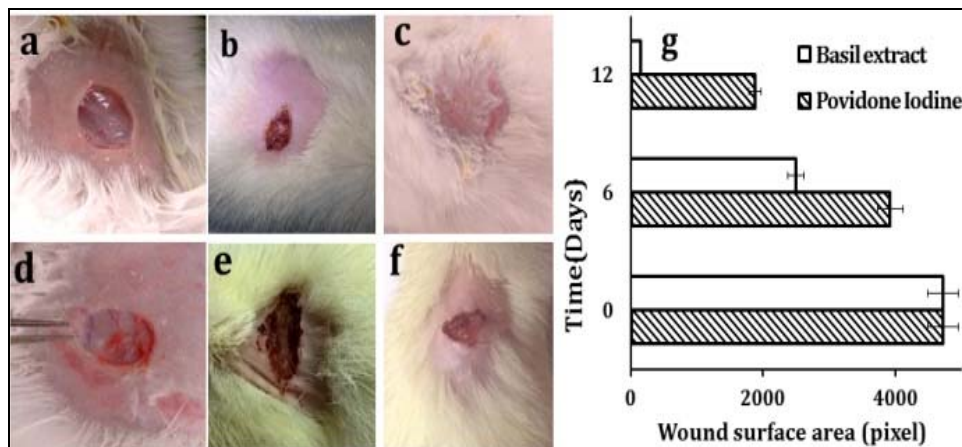


Fig 2: (a & d) fresh wound at day 0, (b & c) show *O. sanctum* extract treated wound at day 6 and 12, (e & f) PI remedied wound at day 6 and 12 respectively and (g) wound area change over 12days

3.3 Tissue architecture studies and Immunohistochemistry

3.3.1 Hematoxylin & Eosin (H & E) and Van Gieson's staining (VG)

Fibroblastic proliferation was noticed in both test and control samples on H&E staining (Fig. 3) of day 12. However more evident homogenization of the collagen fibers throughout the papillary dermis and the upper one-third of the reticular dermis were observed in *O. sanctum* extract treated samples which is in congruent with appropriate tissue healing and reorganization. Epithelization was complete and well defined in *O. sanctum* treated samples with evidence suggestive of tissue contracture and restructuring. The outcome was found in congruent with previous studies reporting normal dermal changes of healing [22]. The precise arrangement of collagen fibrils into ordered assemblies is of crucial importance for the mechanical properties of the tissue [5]. VG staining exhibited (Fig.4), two morphologically distinct layers, defined basement membrane and increased deposition of collagen. which can be easily appreciated in *O. sanctum* extract treated samples by day 12 with slack meshwork of thin collagenous fibers in papillary dermis and thick, coarse collagen bundles in reticular dermis [23]. Such organization of collagen bundles was found missing in PI treated wounds in all the samples. A remarkable increase in collagen intensity in the sub-epithelial connective tissue was recorded at the various evaluation points in *O. sanctum* extract treated sample which was missing in PI treated sample (Table1).

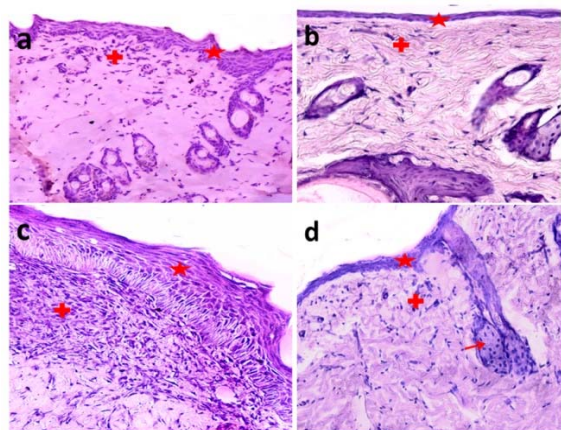


Fig 3: H & E staining image at 20X magnification of *O. sanctum* extract (a & c) and PI (b & d) treated wound of diabetic rat at day, 6 and 12 days respectively.(Star, cross and arrow represents epithelium, fibroblasts and hair follicle respectively

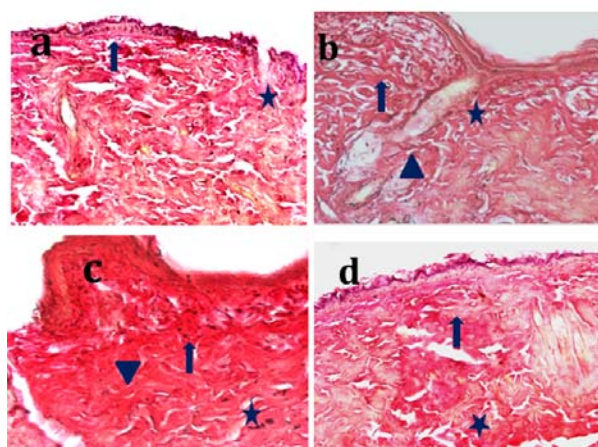


Fig 4: (a & c) shows van Gieson's staining of wound biopsy tissue after *O. sanctum* extract treatment and (b & d) after PI treatment on 6 and 12 days respectively. (Arrow indicates papillary dermis and star indicates reticular dermis and arrow head hair follicle)

3.3.2 Semi-quantitative evaluation of histological and immuno-histochemical features

The VG intensity was evaluated grounded on a visual perceptual gauge guided by pathologists based on standardization scheme (Fig 5.b). Fig 4a shows the process of VG scoring intensity documentation with the aid of three points L1, L2 and L3 along the 200 μm from basement membrane (sub-epithelial area). Result shows the increased deposition of the collagen in *O. sanctum* extract wound depicted by increased VG intensity results when compared to PI treated one ($p \geq .05$). This is supported by previous studies on wound healing [24]. Basement membrane (BM) thickness in *O. sanctum* treated was found to be 1.39 ± 0.48 in comparison to that of 1.18 ± 0.24 seen in PI treated one depicting poor collagen formation. Epithelial thickness of the sections from *O. sanctum* extract treated wound was 32 μm and 53 μm respectively on 6th and 12th day in comparison to 15 μm and 18 μm of PI treated wound, which evidences the efficiency of the earlier in swift epithelization and maturation of the wound. Apart from organizational properties, the dermo-epidermal basement membrane carry gate-keeping utilities monitoring cell traffic of bioactive molecules in both directions and serves as a reservoir for controlled release of a variety of cytokines and growth factors [25]. This plays a vital part during physiological repair processes following injury and in chronic inflammatory conditions like diabetes.

O. sanctum with its inherent analgesic, anti-inflammatory, antimicrobial, antioxidant, anti ulcerogenic and chem-immuno modulatory aids in rejuvenation of injured basement membrane and connective tissue in tissue injury as seen in this study. Flavonoids sequestered from *O. sanctum* foraged free radicals in vitro protecting against cellular injury and showed anti lipoperoxidant activity *in vivo* at very low concentration and this free radical scavenging action of the flavonoids aid in wound healing [24, 26].

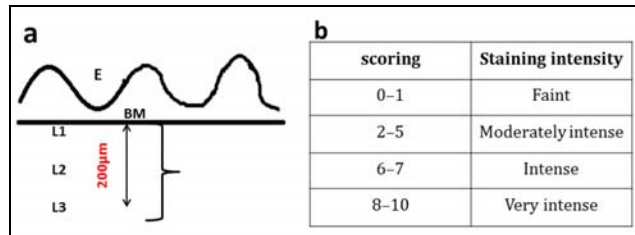


Fig 5: (a) Depicts the methodology adopted to generate data on VG intensity. L1, L2 and L3 are the points considered for intensity scoring where L1 is present just below the basement membrane (BM) whereas P3 lies at a distance of 200 µm from BM and L2 lies in between the two (b) represents staining intensity scoring scale.

Table 1: VG intensity scoring of three evaluation points L1, L2, L3 along with F value in *O. sanctum* and PI treated samples.

Intervention	L1	L2	L3	F Value	Time schedule
Basil Extract	6.94±0.1	6.3±0.18	6.5±0.31	15.5	Day6
	8.36±0.28	7.65±0.23	8.5±0.23	13	Day12
Povidone Iodine	5.85±0.2	6.01±0.21	6.21±0.19	13.5	Day6
	6.96±0.1	7.2±0.2	7.45±0.2	10	Day12

O. sanctum extract treated wound demonstrates systematized collagen III distribution at 6 days with progressive decrease in expression and simultaneous increase in collagen I which contributes to the wound strength by the end of 12 days of study (Fig. 6) [27]. Semi-quantitative analysis shows 54.18% and 63.49% decrement in collagen III expression by day 6 and day 12 of honey treated wound whereas PI treated wounds shows relatively less replacement of Collagen III

(13.26% at day6, 35.14% at day 12). Collagen I expression increases over 12 days simultaneously akin to normal wound healing. Collagen I to collagen III ratio improved from 2.6:1 to 3.9:1 in *O. sanctum* extract treated wound akin to normal skin by 12 days whilst Collagen I to collagen III ratio changed from 1.3:1 to 2.2:1 in *O. sanctum* extract treated wound owing to poor collagen I formation which is vital for mechanical integrity (Fig 6.c).

Type III collagen is the primary one to arise during wound healing, acting as bridge followed by type I collagen which in combination with type III during tissue reorganization to build a concrete support facilitating wound healing while circumventing the scar tissue formation [27, 28]. Type III collagen supposedly deliver a grid of adhesive macromolecules proficient in orienting the relocation of endothelial cells to configure the granulation tissue; thereafter, this molecule is gradually replaced by type I collagen the major molecule responsible for providing tensile strength to the tissue, so that the scar acquires mechanical stability. Rapid replacement of type III for type I collagen is characteristic of normal healing. Diabetes is allied with a marked diminution in collagen production, when compared with non-collagen protein and cumulative results of these noticeable changes in collagen production may back chronic connective tissue complications in diabetes and poor wound healing as per previous studies [29, 30].

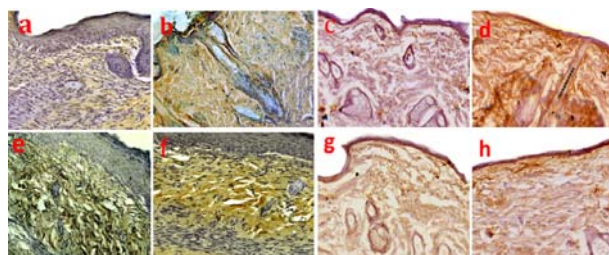


Fig 6: (a & e) and (c & g) shows immunohistochemistry images at 20X magnification of Collagen I expressions in *O. sanctum* extract PI treated wound respectively on day 12. (b & f) and (d & h) shows immunohistochemistry images at 20X magnification of Collagen III expressions in *O. sanctum* extract and PI treated wound respectively on day 12.

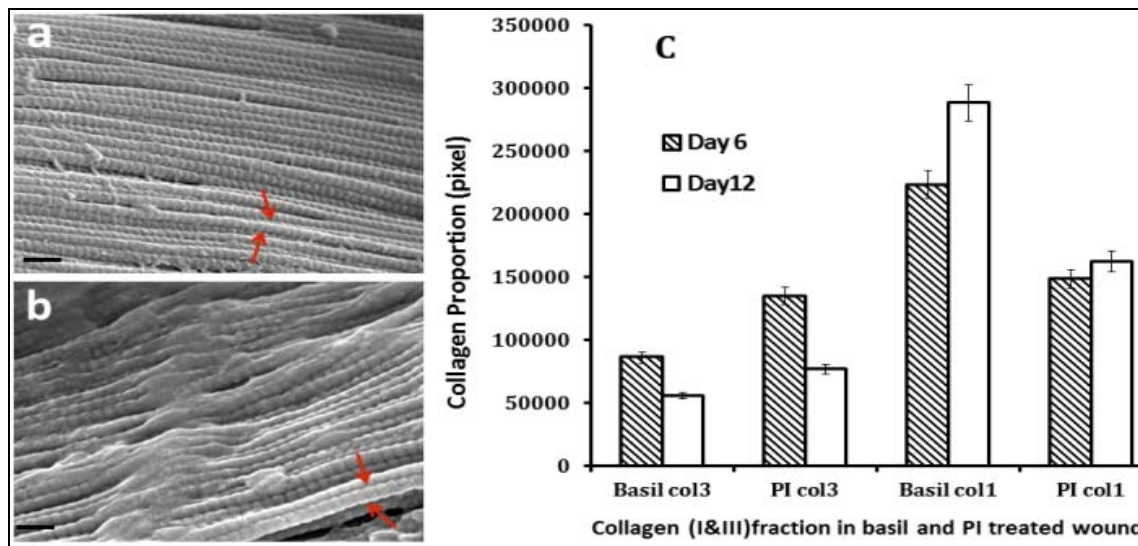


Fig 7: (a) and (b) show SEM images of collagen fibrils in extracellular matrix of *O. sanctum* and PI treated healing wound in diabetic rabbit on day 12 (Scale bar = 200nm). (c) shows semi-quantitative analysis of Collagen I & III expression followed by replacement in PI and honey treated wound

3.3.3 Scanning Electron Microscopy (SEM) analysis

As shown in Fig 7, collagen fibrils appeared to be swollen on the day 12 with mean radius of 129 ± 4 nm on day 12 in tissue biopsy from PI treated wound margin. However the *O. sanctum* extract treated wound sample displayed relatively improved organization of collagen fibrils after 12 days of study with mean radius of 104.1 ± 3 nm. The collagen fibrils were randomly and loosely arranged in the matrix in the PI treated group in comparison to the compact arrangement in *O. sanctum* extract treated one. As described earlier protracted acquaintance with hyperglycaemia fallouts in lack of ordered collagen fibrils in dermal tissue and basement membrane resulting in poor wound strength. It also endorses non-enzymatic glycation directing to the build-up of end-products, which attributes to diabetes complications. In long-lasting glucose intolerance, collagen becomes increasingly stiffer, owing to glycation dependent crosslinks^[31, 32].

4. Conclusion

Diabetes is a part of chronic metabolic syndrome with multifaceted effects in human body, attributing to the huge health care economy burden. Flaws in immunity, collagen formation and glycation seen in diabetic population results in poor wound healing and increased wound infection. A major chunk of the world population relies on traditional medicines for the health care especially in developing world. In the present study, alcoholic extract of *O. sanctum* is found to be superior to povidone iodine in preventing infections suggested by regular wound swab culture and clinical examination. Augmented wound healing is seen in the *O. sanctum* extract treated wounds evidenced by tissue architecture studies showing increased regular and compact collagen production with favorable collagen I to collagen III ratio. Promising elementary evidences for wound healing properties are suggested by clinical, histo-pathological and immuno histochemical studies. Thus study highlights the vitality of widely available *O. sanctum* extract with scarcer side effects as a cost effective and safe natural alternative medicine with improved outcomes for rapid diabetic wound healing in comparison to synthetic counterparts. However, it also warrants further experimental substantiation toward transformation of the natural product like *O. sanctum* extract for evidence based translation from bench to bedside.

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