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ORIGINAL ARTICLE

In vivo Evaluation of *Acalypha indica* Extract Incorporated Electrospun Guar/PVA Nanofibrous Mats for Use as Active Wound Dressing Material

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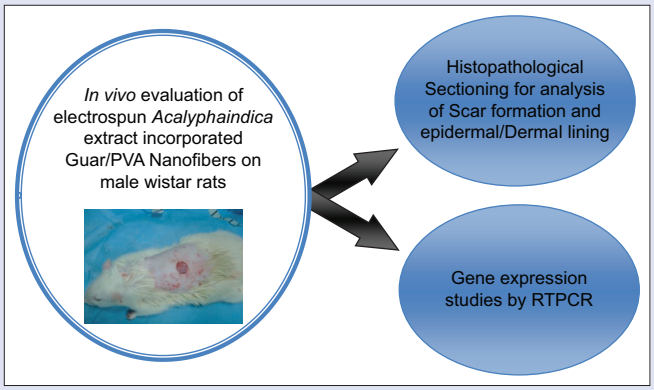
ABSTRACT

Aim: This study aimed to evaluate *Acalypha indica* extract incorporated electrospun guar/PVA nanofibrous mats for use as active wound dressing material by *in vivo*. **Background:** *A. indica* is a well-known plant for its wound healing properties and has been used for decades by tribes of Asia and Africa. **Materials and Methods:** *In vivo* evaluation of fabricated electrospun nanofibrous mats with 5% *A. indica* incorporated 3:7 of 1 wt% guar/10 wt% PVA by excisional wound model on rats followed histopathological and gene expression analysis. **Results:** The results indicated that guar/PVA nanofiber loaded with 5% of *A. indica* extract showed higher performance and synergistic effect for wound healing applications by remarkably capable of healing the wounds up to 97.5% after 14 days post-treatment periods. Histopathology and gene expression results revealed complete closure of wound with narrow scar, complete epidermal–dermal lining formation, maximum upregulation of wound healing related genes of involucrin, collagen IV, integrin 6-alpha, E-cadherin, matrix metalloproteinase-9, and vascular endothelial growth factor.

Key words: *Acalypha indica*, guar, nanofibers, wound dressing, wound healing

SUMMARY

- *In vivo* evaluation of the dressing on male Wistar rats enhanced the contraction of wounds with the complete formation of dermal and epidermal layers and also with narrow scarring
- Upregulation of the wound healing related genes of vascular endothelial growth factor -F, transforming growth factor-β, E-Cadherin, Involucrin, Integrin 6-alpha, matrix metalloproteinase (MMP)-2 and MMP-9 was observed
- The dressing with *Acalypha indica* extract (polymer blend with 5% of *A. indica*) holds promise to be developed as a uniquely indigenous primary aid for acute wound care commercially, shedding light on the therapeutic application of plant extract-based dressing for wound healing applications.



Abbreviations used: PB: Polymer blend; PB5Ai: Polymer blend with 5% of *Acalypha indica;* HDF: Human dermal fibroblasts; RTPCR: Real-time polymerase chain reaction

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INTRODUCTION

Skin is the largest and outermost organ of the body that protects the internal organs from the pathogenic environment.[1] Chemical, physical, thermal, microbial, and immunological abuse causes damage to the skin. Depending on the physiology, nature, and severity, it is categorized into acute and chronic wounds.[2] Wound healing is an immediate immunological response that occurs in an orderly manner with four overlapping sequential time-dependant phases of hemostasis, inflammatory, proliferative, and remodeling phase. Local factors such as oxygenation, infection, foreign bodies, and systemic factors such as stress, age, obesity, diabetes, therapeutic drugs, prolonged medication, consumption of alcohol, and smoking have action on delayed wound healing.

Traditional systems of medicine based on the observations and practices over decades use different plant materials for the treatment of wounds. The plant extracts involve in the process of disinfection and debridement.[3] *Acalypha indica* is one such traditionally acclaimed plant that is widely used to treat skin-related ailments. Scientific reports by Reddy *et al.*,[4] Ganeshkumar *et al.*,[5] and Ibrahim *et al.*[6] confirm its wound healing property *in vivo*. The plant extract is currently available in aqueous and ointment forms which do not have a stable shape and maceration to the surrounding tissues occurs if uncovered. They cannot be applied directly to exudating wounds. Their physical forms limit their permanency. Thus

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to avoid such complications, fabricating the plant extract material as a wound dressing is popular in recent trends.

Wound dressings are widely used in recent times due to their relatively low cost, ease of use, effectiveness to clean, cover, and protect from the pathological environment. Traditional dressings such as cotton wool, gauze, and bandages are still the most commonly used materials for wounds and burns, which are restricted due to their stability problems. They are thus replaced by modern dressings which can be designed to act as drug delivery systems incorporating therapeutic agents/natural products. They can be developed into fibers, thin films, foams, gels, and so on with the use of natural or synthetic polymers or from the combination of both.[7,8]

We have earlier developed 3D nanofibrous mats with the combination of natural and synthetic polymer composite guar/PVA incorporated with *A indica* extract. The wound dressing material turned out to have ideal characteristics in terms of structural, mechanical, and thermal endurance. Assessment of its applicability through antimicrobial activity, HDF proliferation, and live-cell imaging assay demonstrated significant antimicrobial activity along with ample sustenance for HDF proliferation, making it an ideal wound dressing material for epidermal reconstruction.[9]

In this chapter, we have evaluated the effectiveness of *A. indica* incorporated guar/PVA nanofibrous mats on rat excisional model by comparing with the commercial wound dressing sterizone-ST and gauze bandages in accelerating wound healing properties.

MATERIALS AND METHODS

PVA and citric acid were procured from Thermo Fisher Scientific (Massachusetts, USA); guar gum was procured from Sigma–Aldrich (Missouri, USA); and the aerial parts of *A indica* belonging to Euphorbiaceae family were collected from Kayathar township of Tuticorin district and authenticated by Dr. V. Chelladurai – Botanist, Government Siddha Medical College, Palayamkottai.

Sample preparation

Polymer blend (PB) nanofibers were prepared by 3:7 mass ratio of 1 wt% of purified guar and 10 wt% of PVA dissolving it in distilled water at 50°C. Polymer blend with 5% of *A. indica* (PB5Ai) nanofibers were prepared by adding 5% of *A. indica* based on the total weight of the polymers to the PB solution. Then finally, citric acid powder was added into the solution (with and without *A. indica*) at 5 wt% by the total weight of the solution.

The polymer solutions (PB, PB5Ai) were fed into a 10 mL standard syringe attached to a 21G stainless steel needle using a syringe pump. Flow rate of the solution was set as 0.5 mL/h (KL602 single-channel syringe pump; KellyMed). High voltage of 20 kV was applied to the polymer solution and the fibers were collected on an aluminum foil wrapped collector kept at a distance of 18 cm from the needle tip. The fibers collected were thermally cross-linked by incubating at 40°C for 48 h.[9]

In vivo efficacy study on male Wistar rats

The *in vivo* efficacy study was performed after getting the approval from Institutional Animal Ethics Committee of Sri Ramachandra Institute of Higher Education and Research (Approval No. IAEC/54/SRU/600/2017). Male Wistar rats weighing 150–200 g were housed under standard conditions. Each rat was anesthetized and the excision wound was created on the shaven back of the rat by cutting out a 2 cm × 2 cm piece of skin. Wounds were sterilized and animals were tested with nanofibrous mats (PB, PB5Ai) and compared with the standard dressing (Sterizone-ST) [Table 1]. Animals under disease control group were maintained without any wound dressing throughout the study. The wounds were routinely examined and the position of the dressings was monitored. The wound contraction areas were recorded by scaling with Vernier caliper for 0, 4, 7, and 14 days for the control, standard, and test rats [Figure 1].[10] On the 14th day, animals were sacrificed and tissues at the wounded area were collected for histopathological and gene expression studies.

Table 1: Details of animal groups used for *in vivo* efficacy study

Group number	Treatment	Number of animals
1	Disease control	6
2	Standard (sterizone – ST)	6
3	Test – 1 (PB)	6
4	Test– 2 (PB5Ai)	6

PB: Polymer blend; PB5Ai: Polymer blend with 5% of *Acalypha indica*

Histopathological studies

At the end of the experimental period, rats from each group were euthanized using CO_2 asphyxiation. Specimens of skin from healed wounds of each rat were taken and fixed in a 10% neutral buffered formalin solution. The fixed tissues were processed, embedded in paraffin wax, sectioned approximately at 3–4 μ thick, and stained with hematoxylin and eosin for histopathological examination.

Gene expression studies-real-time polymerase chain reaction

Real-time polymerase chain reaction (RTPCR) was performed to study the upregulation of genes by the wound dressing materials, in the process of wound closure. Specimens of skin were collected in RNA later and immediately stored in liquid nitrogen. The tissue samples were homogenized and cells were collected. The RTPCR was then performed, as reported in Jenifer *et al.*[11] (Genes used: Collagen IV, involucrin, interleukin-1, interleukin-6, integrin alpha-6, E-cadherin, matrix metalloproteinase [MMP]-2, MMP-9, TNF-alpha, smad-3, vascular endothelial growth factor [VEGF], and transforming growth factor [TGF]-beta).

RESULTS

Percentage of wound closure

The percentage of wound closure for disease control (G1), standard control (G2), polymer nanofiber (PB) (G3), polymer nanofiber with *A. indica* extract (PB5Ai) (G4) on days 2, 5, 7, 10, and 14 is presented in Table 2. Polymer nanofiber with *A. indica* extract (G4) had a significant effect on wound closure percentage with respect to Group 1 ($P < 0.05$). When compared with Group 1 for percentage of wound closure from day 2 to day 14 (11.53 ± 0.30% to 93.02 ± 0.36%), Group 4 had a gradual increase in percentage of wound closure from 29.06 ± 0.28% to 97.53 ± 0.17%, respectively. Results revealed that treatment with *A.indica* incorporated dressing (Group 4) resulted in a much faster contraction of wound. Extract-treated wounds were found to heal much faster as indicated by the period of epithelisation which was significant at $P < 0.005$*.

Histopathological analysis

Scar formation

Figure 2 summarizes the wound healing pattern in the experimental animals. Scar tissue formation is a result of irregularly packed collagen fibers and incomplete dermal formation in different degrees. The disease control (Group 1) showed wider scar formation, while the Group 2, the

Table 2: Analysis of variance for percentage of wound closure

Group	Day 2 (%)	Day 5 (%)	Day 7 (%)	Day 10 (%)	Day 14 (%)
Disease control (G_1)	11.53±0.30	29.34±0.23	47.27±0.25	77.18±0.30	93.02±0.36
Standard control (G_2)	12.43±0.24	29.42±0.16	46.56±0.20	76.96±0.27	92.61±0.23
Test – 1 (PB) (G_3)	17.37±0.22*	32.40±0.22*	47.32±0.23	78.02±0.14	93.01±0.39
Test - 2 (PB5Ai) (G_4)	29.06±0.28*	43.99±0.23*	74.75±0.70*	83.57±0.31*	97.53±0.17*

*Significant (P<0.05). PB: Polymer blend; PB5Ai: Polymer blend with 5% of *Acalypha indica*

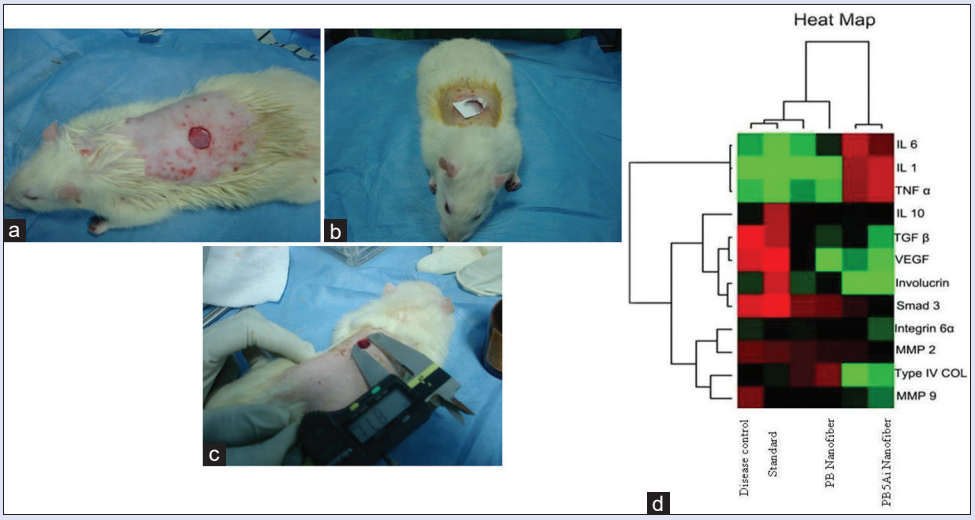

Figure 1: Photographic images of (a) Excision wound created rat, (b) Placement of nanofibers as wound dressings, (c) Measurement of wound closure using a Vernier caliper, (d) Heat map showing the genetic expression of healed animal tissues using real-time polymerase chain reaction

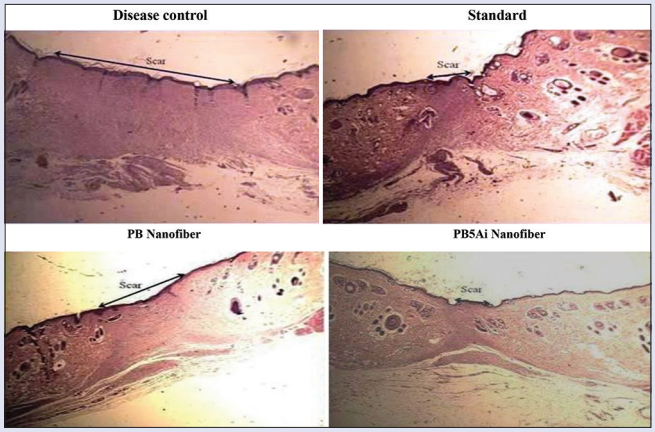

Figure 2: Histopathological sectioning of scar formation after wound healing

standard, exhibited normal scar formation. There were wider scars in the polymer nanofiber – PB (Group 3). Narrow scars were seen in Group 4, which implicates *A. indica* modulation in improved wound healing.

Epidermal and Dermal lining

Figure 3 summarizes the histopathological view of complete formation of epidermal and dermal layers in PB5Ai nanofibers which was similar to that of the normal skin. Although the wound closure was 93.5% in disease control group, the layers were filtered with inflammatory cells.

Gene expression analysis-real-time polymerase chain reaction

PB5Ai showed downregulation of interleukin-6 (IL-6), IL-1, and TNF-alpha which are primarily involved in the pro-inflammatory and anti-inflammatory response. Minimal upregulation of MMP-2 and smad-3 and maximal upregulation of involucrin, collagen IV, TGF-beta, integrin 6-alpha, E-cadherin, MMP-9, and VEGF were observed. PB nanofiber showed upregulation of all genes except collagen and smad-3 which are the primary genes in the formation of epidermal structure. Similar to PB5Ai nanofiber, the standard also exhibited downregulation of IL-6, IL-1, and TNF-alpha [Figure 1d].

DISCUSSION

Human skin is the largest organ of the body which primarily serves as a protective barrier against the environment. An injury to the tissues of the body caused by any physical means or any interruption to the tissues is defined as "wound" and restoration process of these damaged tissues is termed as "healing or wound healing." Traditionally acclaimed wound healant herbs such as *A. indica* are classically applied extemporaneously as aqueous pastes. However, aqueous extracts serve as a source for the growth of micro-organisms which is attributed to the enzyme polyphenol oxidase that degrades polyphenols in water.[12] Thus for modern-day proprietary herbals, the dried plant material is solvent extracted with ethanol and developed as a wound dressing for potent effectiveness and longer shelf life. Conventional topical dosage forms are not preferred as they immediately dry and external covering is needed to avoid maceration of surrounding tissues.

One of the approaches to develop a wound dressing material is by fabricating biodegradable nanofibers with biocompatible polymers and plant extract. As reported in Jenifer *et al.*, 2018.[9] With a 3:7 mass ratio of 1 wt% guar: 10 wt% PVA, and by 2%, 5%, and 10% of *A. indica,* respectively, three plant extract incorporated 3D nanofibrous mats were developed by optimizing the polymer combination and concentration based on the nondissolution criteria and the resultant product turned out to be an ideal dressing by showing its ability to remove the excess

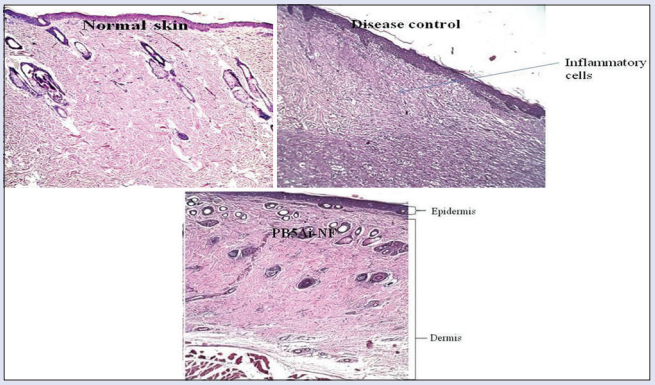

Figure 3: Histopathological sectioning of skin for analysis of formation of epidermal and dermal layer after wound healing of normal skin, disease control skin, and PB5Ai nanofiber-treated skin

wound exudates, perfect tensile strength that matches the human skin, thermally stable, and ability to provide protection against bacterial infection.

An ideal dressing should also enhance epidermal reconstruction and minimize scar formation. So in this chapter, the *in vivo* efficacy study was carried and the results established faster wound healing of PB5Ai nanofibers in male Wistar rats. The base polymer (PB) was checked for its dermal toxicity. No signs of either dermal or systemic toxicity were observed in any of the female Wistar rats. Nil mortality and morbidity were observed during the course of the study.[13] The wound contraction was determined by the planimetric method and the period of epithelialization, i.e., the total number of days taken for complete healing of wound without any raw wound left. Rats by itself have a drawback of faster wound contraction, as they have "loose skin." The loose skin allows wound contraction to play a significant role in closing rat skin wounds, whereas human have tight skin.[14] However, the core lies in the minimal scar and complete formation of dermal and epidermal lining. PB5Ai brought about similar activity by closing the wound at 97.5%. Although closure of the wound was faster even in the disease control group, the histopathological analysis revealed a wider scar filtered with inflammatory cells. However, PB5Ai nanofibers revealed the complete formation of dermal and epidermal layers, blood vessels formation along with narrow scarring. RT-PCR analysis revealed the maximal expression of TGF-β, involucrin, integrin 6-alpha, E-cadherin, MMP-2, and MMP-9 which play significant roles in cellular proliferation, cell differentiation, tissue formation, controlled cell proliferation, and structural formation of hemidesmosome. Enhanced expression of VEGF protein signifies its facilitatory role in blood vessel formation associated with wound healing.

CONCLUSION

In this study, the performance of the effect of PB5Ai nanofibers on wound healing activity was evaluated. *In vivo* studies on rat excisional wound model for representative nanofibrous wound dressing samples with and without *A. indica* extract exhibited that simultaneous use of *A. indica* and guar/PVA as a tissue cultured polymer with a nanofibrous structure can produce an increased rate of re-epithelialization and minimal scar formation with the upregulation of wound healing-related genes of TGF-β, involucrin, integrin 6-alpha, E-cadherin, MMP-2, and MMP-9 after 14 days posttreatment. Therefore, the results suggest that the PB5Ai nanofibers could be used for tissue engineering applications and can be further carried out for clinical trials.

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Conflicts of interest

There are no conflicts of interest.

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