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Analysis of the Efficacy of Topical Aqueous Creams Containing Azadirachta Indica Leaf Extract for Healing Wounds

Dr T K V Kesava Rao[1], M Vineela[2]
Professor & Principal[1], Assistant Professor[2]
PYDAH COLLEGE OF PHARMACY, KAKINADA YANAM ROAD, PATAVALA

Abstract

Background: Wound is one of the health indispositions with adverse socio-economic repercussions on the sufferer and those around them. Crude aqueous extract of Azadirachta indica leaves (AEAIL) preserves demonstrated potentials for wound healing. Developing the AEAIL into a topical aqueous cream might boost its effectiveness in wound therapy. purpose: The purpose of this research was to produce aqueous topical creams containing different concentrations of AEAIL as bioactive components, assess their stability and wound healing activity in male Wistar rats using hydroxyproline (HXP) as a biochemical marker.

Materials and methods: Creams containing 1.0, 1.5, 2.0 and 3.0 % w/w of AEAIL were made, evaluating their stability up to 14 days and measuring their wound healing capabilities in male Wistar rats using DMSO, cholesterol and distilled water as controls.

Results: All the batches of creams were stable in colour, pH, viscosity, etc. and demonstrated wound healing effects with the animals treated with the cream containing 1.5 % w/w of AEAIL having the greatest tissue HXP level ($p > 0.05$). The tissue HXP levels in the animals treated with DMSO, cholesterol and distilled water were lower than those of the test creams ($p < 0.05$). There was substantial marginal variations in percentage difference of their HXP level compared to those of the test creams ($p < 0.05$).

Conclusion: The aqueous extract of Azadirachta indica leaves manufactured as aqueous cream was stable and preserved its wound healing properties. This novel solution might potentially be employed in the treatment of bodily injuries.

Key words: Wound healing; Aqueous cream; *Azadirachta indica* leaves; Bioactive ingredient; Hydroxyproline; Wistar rats

Introduction

Wounds are damages to the outer body covering which disrupt the other soft flesh [1]. It causes social and financial impairment to the affected individual and others around them [2]. They may be initiated by physical, chemical, thermal, microbial or immunological abuse to the tissue [3,4] and could ordinarily be defined considering their depth, healing time, the progression of restoration, underlying pathology, the associated risk of mortality and the effect on the quality of life of the victim [5,6]. A ripped, sliced or punctured outer bodily cover is characterized as an open wound. If a blunt force discomfort brings about a bruise, the consequence is characterized as a closed wound. Those wounds classified as a burn are triggered by fire, heat, radiation, chemicals, electricity, or sunlight [3,4]. Restoring an injury to the body is a prolonged and complicated progression of tissue healing and transformation in reaction to an injury involving a complex series of cellular and biochemical reactions to pave way for the restoration of the injured part of a body to the re-establishment of the fundamental and serviceable constitution of the tissues as it was. It comprises continuous cell-cell interface and cell-matrix interactions that let the method to continue in varied related segments and procedures involving inflammation, wound contraction, re-epithelialization, tissue re-modelling and formation of granulation tissue with angiogenesis. The levels of restoration of an injured body usually advance in an anticipated time frame until healing is accomplished, of which if it fails, the expected healing may not be achieved, and may lead to whichever, a long-lasting wound like a venous sore or pathological damaging such as a keloid scar [7]. The initial phase of wound healing regulates bleeding [8,9]. The constriction of the vascular system, platelet migration and production of coagulated fibrin restores haemostasis immediately after vascular injury and provides space for an extracellular network for cell migration. With the aid of this, mediators of wound healing recruit inflammatory cells to the location of wound enabling the following stage of inflammation [10]. This second phase interacts with the previous phase involving haemostasis and clotting and is beginning in a few hours following the injury. This phase is essentially separate from the increase of leukocytes and macrophages [9,11].

Macrophages enter into the damaged location, release growth factors like a platelet, encouraging the creation of new connective tissue or granular tissue [9]. Macrophages similarly contribute in the resolution of inflammation and stimulate tissue regeneration, allowing the change from the inflammatory to reparative phases of proliferation and remodelling, happening within three weeks from the day of the injury to the skin. The third phase, proliferation is distinguished by granulation tissue production, re-epithelialization of the wound surface and contraction of the wound edges [8,11]. Granulation tissue includes macrophages, fibroblasts and immature collagen, all recognized to encourage granulation tissue production. Simultaneously, blood vessels will encourage capillary development. Fibroblasts near the surface of the wound spark the synthesis of collagen, one of the essential elements of the extracellular matrix. The fourth stage of wound healing might continue for a very long period and consist of the restructuring of collagen fibres to form new skin [8,11]. Fresh skin might progress less than a fourth of its potential strength within three weeks from the day of the damage and seldom ever attains the strength of the original skin [9].

Innumerable plant concoctions have at one point or the other been applied in the care of bodily injuries. Plant-based extracts used in mending wounds stimulate blood clotting, combat infection and expedite the healing of wounds. Phytoconstituents originating from plants need to be explained and explored for their appropriateness for treatment of wounds [1].

Azadirachta indica or neem which has been effective in traditional medicine has been utilized therapeutically for wounds, incisions and additional skin diseases. Its flavonoids sustain antioxidant function, guarding against free radicals that harm cells and tissues. Its tannins enhance wound healing [12-14]. A. indica includes variations of phytoconstituents [15]. Research records suggested that ethanolic or methanolic extracts of A. indica leaves contains wound healing potential [16, 17]. A quantification of the phytoconstituents of the AEAIL has been undertaken using the GC-FID methods [18]. The phytomedicinal and the nutraceutical advantages of the AEAIL has also been proven [19]. A recent study using hydroxyproline (HXP) as a biochemical marker to evaluate the level of collagen formation in wound healing showed that the AEAIL possesses wound healing activity with the optimal effective concentration for wound healing in male Wistar rats established to be 1.5 % w/v of its crude extract [18]. The goal of this research was to construct an aqueous cream containing the AEAIL and to test its stability and retention of wound healing potentials in male Wistar rats. The degree of wound healing activity of the AEAIL contained in the topical creams will be examined based on the pace of wound contraction, but, notably the amount of tissue HXP detectable in the area of healed wound in male Wistar rats. HXP is a non- essential amino acid derivative generated via post-translational protein modification by hydroxylation of the amino acid, proline by the enzyme prolyl hydroxylase which needs vitamin C as a co-factor. HXP is a crucial component of the protein, collagen and serves a critical function in the integrity of the collagen triple helix [20].

Materials and methods

Materials

The following materials were employed for the investigations as purchased and comprise hydroxyproline test kit (Elabscience, China), dimethyl sulphoxide (DMSO) (Sigma-Aldrich, USA), cholesterol (Molychem, India), emulsifying wax, liquid paraffin and soft paraffin (Kerax, UK).

Methods

Collection and extraction of the sample of Azadirachta indica leaves

Fresh neem leaves used had been identified by a Taxonomist and placed in the University of Port Harcourt herbarium (voucher number. EH/P/070) as described by Ugoeze et al [18,19]. The technique of sample collection and processing as also published by Ugoeze et al [18,19] was employed.

Formulation of aqueous cream comprising aqueous extract of A. indica leaves

The aqueous topical creams comprising varying strengths of the aqueous extract of Azadirachta indica

leaves (AEAIL) were produced following the formula in Table 1.

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Table 1: Formula for the preparation of topical creams containing different concentrations of AEAIL

Ingredients	Batches / Composition (% w/w)			
	A	B	C	D
AEAIL	1.0	1.5	2.0	3.0
DMSO	5.0	5.0	5.0	5.0
Cholesterol	10.0	10.0	10.0	10.0
Glycerol	5.0	5.0	5.0	5.0
Emulsifying ointment	20.0	20.0	20.0	20.0
Water, q.s.	100.00	100.00	100.00	100.00

Evaluation of the stability of creams

The cooled creams were examined visually with the naked eye for their look and color. Their consistency was examined by rubbing a tiny part of it between the fore and first fingers while their homogeneity was measured in terms of creaming and phase separation. A known quantity of each batch of cream was put in a wide-mouthed plastic container and kept at -5°, ambient temperature and 40°C correspondingly. The color, look, consistency and homogeneity of these samples were monitored everyday for 14 days for any changes. The pH of the batches of the creams was measured using the pH meter (Ultrameter II, 6PFC E; Myron L, UK). They were maintained at ambient temperature while their pH was checked every other day for 14 days [21]. The creams were also examined for their spreadability, a process established by inserting a known amount of each cream between two slides while a 100.0 g weight was placed on it for 10 s the distance displaced by the slides was documented.

Viscosity

The viscosity of the creams was tested every other day for 14 days using a Brookfield viscometer (Brookfield DV2TLVTJO, USA) connected with spindle no. 62 at ambient temperature.

Evaluation of the wound healing effects of the creams using wound excision procedures in male Wistar rats

Thirty-five adult male Wistar rats weighing 200-250g, sourced from the animal house of the Faculty of Pharmaceutical Sciences, University of Port Harcourt were kept in separate cages to acclimatize for two weeks with free access to standard feed and water all through and in standard conditions of temperature (25-29), relative humidity (55-66%) and natural dark/light cycle. Each rat was anaesthetized by 50 mg/kg ketamine intramuscularly. Shaving, cleaning of the dorsal region and excision of a 1.5 cm × 1.0 cm full-thickness open incision was created [22]. Animal upkeep was typically handled in strong adherence of the ethical criteria of the University of Port Harcourt. The inquiry was done in conformity with the standards for ethical behavior in the care and use of non-human animals in research [23]. All the experimental protocol for the animal research were checked and approved by the Research Ethics Committee of the University of Port Harcourt with approval reference no.: UPH/CEREMAD/REC/MM71/043.

Evaluation of wound healing effects of the aqueous creams utilizing animal model

A total of 35 mature male Wistar rats were split into 7 groups (n = 5). Groups 1- 4 were treated with the creams containing 1.0, 1.5, 2.0 and 3.0% w/w AEAIL correspondingly whereas groups 5-7 were treated with DMSO, cholesterol (2% w/v) and distilled water respectively. DMSO and cholesterol were utilized in the research to separate their influence on

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hydroxyproline synthesis from those of the AEAIL in wound healing. The therapy was carried out daily coupled with the measurement of wound contraction prior to cleaning and administration of treatments until the closure of the wounds. The proportion of wound contraction was estimated using equation 1 below [24].

$$Percentage\ of\ wound\ contraction = \frac{Initial\ wound\ size - specific\ day\ wound\ size}{Initial\ wound\ size} X\ 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Determination of tissue hydroxyproline

At the full healing of most of the wounds, the rats were slaughtered. Tissue bioassay was done using 100 mg of the tissues obtained from the location of the healed wound of the individual rats, mixed to 1 ml of 6 M hydrochloric acid, heated for 6 h and cooled. The pH was corrected to 6.8 while the volume was brought up to 10 ml using distilled water. Each sample was centrifuged and 1ml of its supernatant was used for the assay of HXP level using the HXP kits (Elabscience, China) and conducting the experiment based on the protocols outlined in the manufacturer's manual which is in line with the methods described by Bergman and Loxley [25] after the principle that the oxidation product produced by HXP under the action of an oxidant reacts with dimethylaminobenzaldehyde (DMAB; Ehrlich's reagent) showing a purplish red colour. The HXP was estimated by measuring the absorbance at 550 nm using a UV-VIS spectrophotometer (Jenway 6405, UK). The data were given as µg/g dry weight of tissue.

2.2.6 Statistical analysis

The figures were provided as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was conducted followed by Fisher's Least Significant Difference (LSD) post hoc test to evaluate the degree of significance.

Results and discussion

Evaluation of the aqueous creams

A homogeneous greenish smooth and consistently stable creams were obtained. It is essential to consider a suitable pharmaceutical formulation that enhances the optimal delivery of the active constituent leading to the efficacy of the preparation. Such formulations should be considered as suitable for the management of open wounds, which among other features, should be easily spread with emollient characteristics. The AEAIL was formulated as a cream employing the principles of oil-in-water emulsions [26]. The formula and the constituents employed in the preparation of the batches of creams were shown in Table 1. The incorporation of the emulsifying ointment enhances the stability of the oil-in-water cream formed, establishing the hydrophilic component. Its presence with water and glycerol provide effective emollient and moisturizing effect which enables the reduction of dryness and irritation of the damaged skin as an occlusive barrier is formed on the skin to inhibit the escape of moisture from the skin. Hydration of the *stratum corneum* permits the opening up of intra and intercellular channels for ease of penetration of active ingredients into the cells and injured tissues. An effective topical dermatologic formulation is also certain since water, glycerol and the emulsifying ointment forms the hydrophilic component of the formulation which serves as a vehicle to solubilize and disperse the extract in the non-aqueous phase of the cream and also supports the mixability and penetration of the extract in the hydrophobic component of the skin [27]. The DMSO and cholesterol contained in the formulation act as amphipathic surfactants to provide further stability to the formulation especially imparting hydrophobic and hydrophilic properties to the cream and enhance the stability of the plant extract acting as the active constituent to retain its activity in the environment of the various adjuvants. In addition to its action as an amphipathic

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surfactant, DMSO also acts as a penetration enhancer [28, 29] which is expected to improve the penetration of the plant extract into the tissue to boost activity. Additional aspects of the oil-in-water based cream take account of easy washability and high skin pore occlusion efficiency. Mostly, occlusion of wounds has been recognized to expressively decrease inflammation which amounts to decline in pains and scaring [30]. Decrease of pain and inflammation as well as speeding up of wound healing has been improved with moist healing environment and such conditions have been attained using oil-in-water creams which tallies with an occlusive formulation [31,32].

The stability of the cream is critical to its effectiveness and safety. The appearance and consistency of the formulations were used to assess the stability of the creams as apparent instability may appear as a change in colour and/or consistency. Considering these features, there were no indications of coalescence, change in colour or inconsistency in the creams in the various stress situations they were exposed to. Spoilage or instability in some creams could occur as alterations in pH which gives rise to unwelcome experiences like skin irritation [33]. The influence of storage time on the pH of the creams is shown in Figure 1. A statistically significant variation in the pH of the creams were recorded ($p < 0.05$), with pH decreasing as the concentration of the AEAIL increased from 1.0 – 2.0% w/w ($p < 0.05$) with no statistical difference in the pH of the creams containing 2.0 and 3.0 % w/w of the AEAIL ($p > 0.05$) (Figure 2). The mean pH of 5.760±0.001, 5.750±0.005, 5.740±0.001 and 5.730±0.001 was recorded for the batches of creams containing 1.0, 1.5, 2.0 and 3.0% w/w of AEAIL respectively as at the 14th day of storage. These values, however, are very close to the pH of the skin of 5.7 for an average adult [34]. A stable pH of the respective batches of creams was recorded (Figure 1) as the storage progressed to the 14th day signifying the stability of the creams containing different concentration of extract.

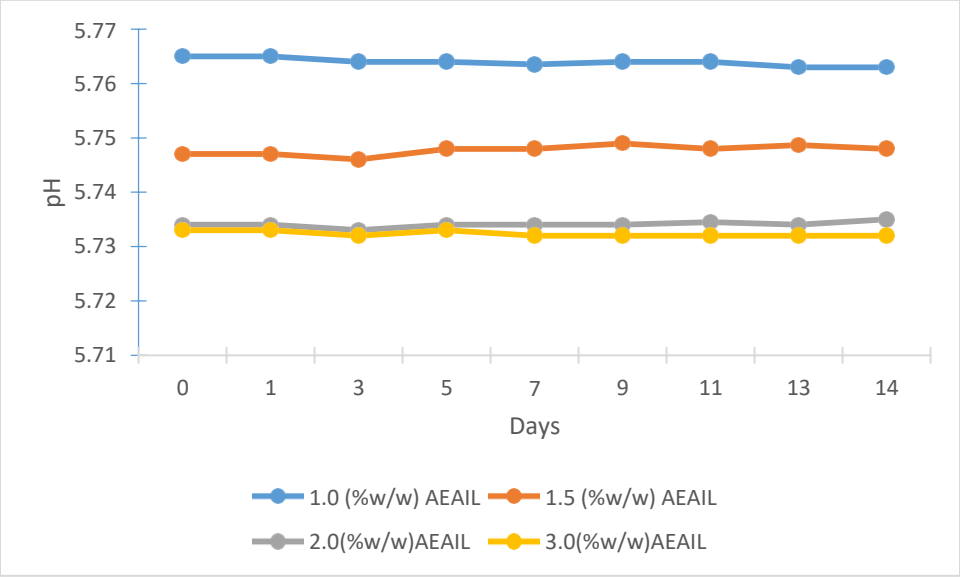

Figure 1: pH of creams following several days of assessment

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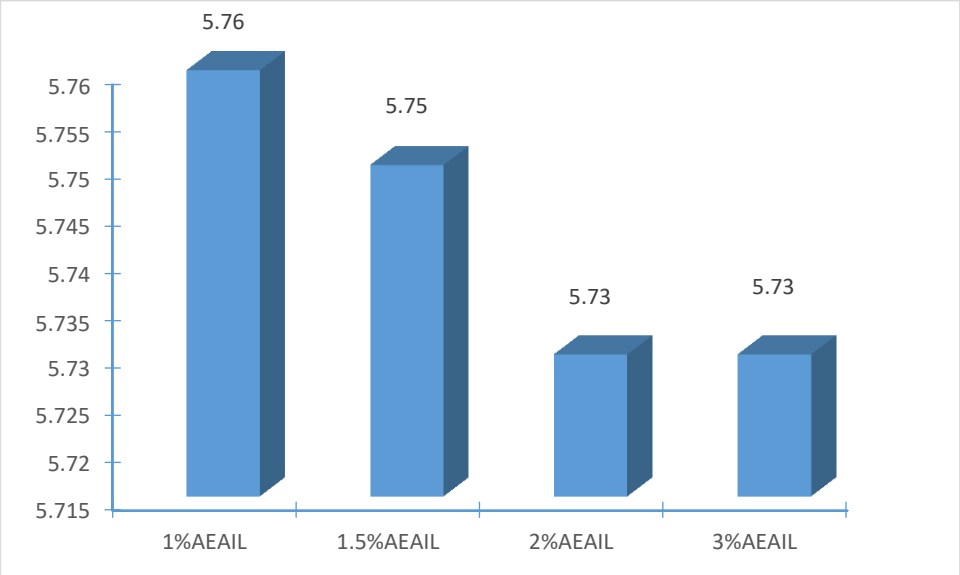

Figure 2: Mean pH of creams containing different concentrations of the AEAIL

The viscosities of the respective batches of creams are presented in Figure 3, showing variations in the viscosities of the batches of creams containing different concentrations of the AEAIL ($p < 0.05$), though there was no consistent pattern of variation of their viscosities. The viscosities of the batches of creams were stable.

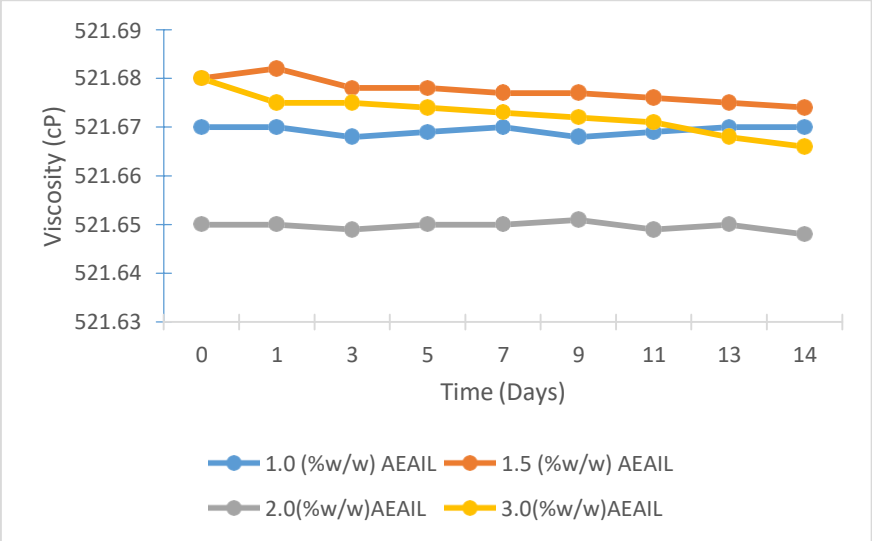

Figure 3: Viscosity of creams following several days of measurement

Evaluation of the wound healing effects of the creams

Figure 4 shows the pattern of contraction of wounds following the treatment with the creams containing 1.0, 1.5,

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and 3.0% w/w of AEAIL and DMSO, cholesterol and distilled water serving as controls. There was a continuous contraction of the various wounds with complete wound closure achieved in the 20, 19, 12, 13 and 20th day of treatment for the creams containing 1.0 and 1.5% w/w AEAIL, DMSO, cholesterol and distilled water respectively. As at the 21st day of treatment, those treated with the creams containing 2.0 and 3.0% w/w of AEAIL showed 98.67 and 98.66% closure respectively.

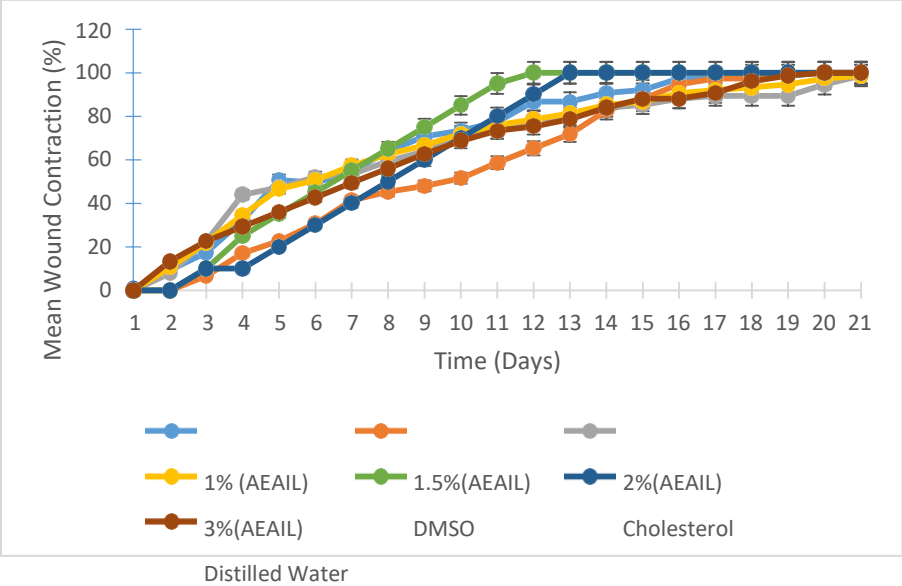

Figure 4: Mean wound contraction following the treatment with creams containing various concentrations of the AEAIL

Results of tissue assay of hydroxyproline

The results of the tissue HXP assay is presented in Table 2 showing the mean tissue HXP levels obtained due to the treatment of various wounds using the creams containing 1.0, 1.5, 2.0 and 3.0% w/w AEAIL and DMSO, cholesterol and distilled water as controls. The results showed that the highest tissue HXP level was obtained from the group of animals treated with the cream containing 1.5% w/w of the AEAIL, though there was no statistical difference in the mean tissue HXP levels of this and those obtained from the groups treated with the creams containing 1.0, 2.0 and 3.0% w/w of AEAIL ($p > 0.05$), but, there was a significant difference in the mean tissue HXP levels for the test creams and the entire control groups ($p < 0.05$) with marked percentage differences in their HXP levels (Table 2). Detection of statistically significant elevated mean tissue HXP in the male Wistar rats treated with the various creams compared to the control groups was an indication of the retention of the wound healing activity of the AEAIL in the presence of the adjuvants employed in the formulation of the creams. This shows that the mean tissue HXP levels detected in the groups treated with the test creams were due to their contents of the AEAIL. In an earlier study, our research team confirmed the wound healing potential of crude AEAIL in male Wistar rats using HXP as a biochemical marker and established its optimal wound healing concentration as 1.5% w/v [18]. The results of the present study have further confirmed that a minimal concentration of 1.5% w/w of AEAIL among its other concentrations used as bioactive

ingredients in the formulation of topical aqueous creams retained its wound healing activity having caused the formation of new collagen as the wound healing progressed as indicated by the highest level of tissue HXP. mechanism of wound healing of the AEAIL may be attributed to its various phytoconstituents [18] based on their anti-oxidant, anti-inflammatory properties, etc.

Conclusion

Aqueous extract of *Azadirachta indica* leaves could therefore be useful as a bioactive constituent in the development of an aqueous topical cream useful in the treatment of body injuries.

Table 2: Difference in the mean tissue hydroxyproline levels of treated groups compared to the control groups (DMSO, cholesterol and distilled water)

Sample	Tissue HXP (µg/g)	Variation of HXP of test samples from controls			Remark
		DMSO	Cholesterol	Dist. water	
1.0% AEAIL	1.5767±0.03	20.82%	24.98%	49.99%	< 0.05
1.5% AEAIL	1.6300±0.09	24.90%	29.20	55.06	< 0.05
2.0% AEAIL	1.4753±0.27	13.05%	16.94	40.34	< 0.05
3.0% AEAIL	1.5594±0.04	19.49%	23.61	48.34	< 0.05
DMSO	1.3050±0.02	-	-	-	-
Cholesterol	1.2616±0.03	-	-	-	-
Dist. Water	1.0512±0.02	-	-	-	-

References

1. 1. Thakur R, Jain N, Pathak R, et al. Practices in wound healing investigations of plants. Evidence-based Complementary and Alternative Medicine 2011 (2011).
2. 2. Bickers DR, Lim HW, Margolis D, et al. The burden of skin diseases: 2004: A collaborative initiative of the American Academy of Dermatology Association and the Society for Investigative Dermatology. Journal of the American Academy of Dermatology 55 (2006): 490-500.
3. 3. Shuid AN, Yusof AA, Anwar MS. The effects of Carica papaya Linn. latex on the healing of burn lesions in rats. Jurnal Sains Kesihatan Malaysia (Malaysian Journal of Health Sciences) 3 (2005).
4. Jalalpure S, Agrawal N, Patil M, et al. Antimicrobial and wound healing activity of leaves of Alternanthera sessilis Linn. International Journal of Green Pharmacy 2 (2008): 141.
5. Fletcher J. Differences between acute and chronic wounds and the function of wound bed preparation. Nursing Standard (through 2013) 22 (2008): 62.
6. Reddy M. Skin and wound care: essential issues in the older adult. Advances in Skin & Wound Care 21 (2008): 424-436.

7. Martin P. Wound healing--aiming for complete skin regeneration. Science 276 (1997): 75-81.

8. Guo SA, DiPietro LA. Factors impacting wound healing. Journal of Dental Research 89 (2010): 219-229.

9. Singer AJ, Clark RA. Cutaneous wound healing. New England Journal of Medicine 341 (1999): 738-746.

10. Boateng JS, Matthews KH, Stevens HN, et al. Wound healing dressings and medication delivery systems: a review. Journal of Pharmaceutical Sciences 97 (2008): 2892-2923.

11. Hanson D, Langemo D, Thompson P, et al. Understanding wound fluid and the stages of healing. Advances in Skin & Wound Care 18 (2005): 360-362.

12. Joshi AR, Joshi K. Ethnomedicinal herbs utilized against skin problems in various communities of Kali Gandaki, Bagmati and Tadi Likhu watersheds of Nepal. Ethnobotanical Leaflets 2007 (2007): 27.

13. Arora N, Bansal MP, Koul A. Azadirachta indica Exerts Chemopreventive Action Against Murine Skin Cancer: Studies on Histopathological, Ultrastructural Changes and Modulation of NF-κB, AP-1, and STAT1. Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics 19 (2011): 179-191.

14. Viji CS, Trikkurmadom SA, Rajalekshmi et al. Collagen—Azadirachta indica (neem) leaf extract hybrid film as a new wound dressing: in vitro research. Int J Pharm Sci Rev Res 32 (2015): 193-199.

15. Govindachari TR, Suresh G, Gopalakrishnan G, et al. Identification of antifungal chemicals from the seed oil ofAzadirachta Indica. Phytoparasitica 26 (1998): 109-116.

16. Barua CC, Talukdar A, Barua AG, et al. Evaluation of the wound healing efficacy of methanolic extract of Azadirachta Indica (Neem) and Tinospora cordifolia (Guduchi) in rats. Pharmacologyonline 1 (2010): 70-77.

17. Osunwoke Emeka A, Olotu Emamoke J, et al. The wound healing benefits of aqueous leaf extracts of Azadirachta indica in wistar rats. J Nat Sci Res 3 (2013): 181.

18. Ugoeze KC, Aja PC, Nwachukwu N, et al. Assessment of the phytoconstituents and best applicable concentration of aqueous extract of Azadirachta indica leaves for wound healing in male Wistar rats. Thai Journal of Pharmaceutical Sciences (TJPS) 45 (2021).

19. Ugoeze KC, Oluigbo KE, Chinko BC. Phytomedicinal and Nutraceutical Benefits of the GC-FID Quantified Phytocomponents of the Aqueous Extract of Azadirachta indica leaves. Journal of Pharmacy and Pharmacology Research 4 (2020): 149-163.

20. National Center for Biotechnology information (2021). Pubchem Compound Summary for CID 5810, Hydroxyproline. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/hydroxyproline [accessed 17.01.21].

21. Akhtar N, Khan BA, Haji M, et al. Evaluation of several functional skin parameters using a topical

cream of Calendula officinalis extract. African Journal of Pharmacy and Pharmacology 5 (2011): 199-206.

22. Suguna L, Singh S, Sivakumar P, et al. Influence of Terminalia chebula on cutaneous wound healing in rats. Phytotherapy Research 16 (2002): 227-231.

23. Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research. Washington DC, USA: American Psychological Association, 2012. Available from https://www.apa.org/science/leadership/care/guidelines. Retrieved June 13, 2019.

24. Kirker KR, Luo Y, Nielson JH, et al. Glycosaminoglycan hydrogel films as bio-interactive dressings for wound healing. Biomaterials 23 (2002): 3661-3671.