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EVALUATION OF WOUND HEALING POTENTIAL OF METHANOLIC *AZADIRACHTA INDICA* LEAVES EXTRACT IN NORMAL AND DIABETIC RATS

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ABSTRACT

The present study was undertaken to evaluate the effect of *Azadirachta indica* leaves extract on wound healing activity in normal and streptozotocin-induced diabetic rats. Methanolic extract ointment was applied on excised wounds in healthy non diabetic and streptozotocin induced diabetic rats. This exhibited significant increase in mean percentage wound contraction and tensile strength in excision and dead Space wound models respectively, in both normal and diabetic rats when compared with control. The extract promoted wound contraction, reduced the wound closure time and induced proliferation of fibroblast as well as angiogenesis and re-epithelialization.

Keywords: Wound Healing, *Azadirachta indica*, Tensile strength, wound contraction

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INTRODUCTION

Diabetes mellitus, which is a morose condition associated with various connective tissue (mainly collagen tissue) abnormalities, is generally associated with delayed wound healing. Delay in wound healing, specifically to diabetic patients, is due to ill function/metabolic behavior/chemotaxis of leucocytes resulting in non-availability of neutrophils and macrophages to the wound[1]. Wound healing is to reduce tissue damage by providing satisfactory blood supply, oxygenation, proper nutrition and moist environment for healing of the wound[2]. It involves the planned and coordinated interaction between extracellular matrix, different types of cells (leukocytes, fibroblasts etc.) and various growth factors such as platelet derived growth factor, epidermal growth factor, fibroblast growth factor etc. Delayed wound healing is producing high economic burden on the society.

Azadirachta indica (Meliaceae), commonly known as Neem is cultivated widely in tropical areas of the world. Its various parts reported to have anti-inflammatory, antipyretic, neuropsychological, antimicrobial, antimycotic, cardiovascular, immunomodulatory, anti-hyperglycemic activity. *Azadirachta indica* used as folk medicine to control leprosy, intestinal helminthiasis, respiratory disorders and constipation[3]. (Kirtikar, 1935)

This study is proposed to evaluate and compare the cutaneous wound healing potential of *Azadirachta indica* in normal and diabetic rats and make inference for the cutaneous wound healing potential by possible "Mode of action" of *Azadirachta indica* extract. There is no significant work done in management of diabetic wound complication of the plant *Azadirachta indica*.

MATERIALS AND METHODS

Plant material and extraction

The leaves of *Azadirachta indica* were collected from the medicinal garden of IPS Group of Colleges, Gwalior (M.P.)in December, 2013 from healthy plants and herbarium was prepared and authenticated by Dr. A. K. Jain, Head, Department of Botany, Jiwaji University, Gwalior(M.P.).Plant leaves was dried under shade and coarsely powdered. Dried powder of drug was then extracted with petroleum ether, chloroform and methanol for 2 h at their boiling point temperatures in soxhlet apparatus. The extracts were concentrated with a rotary evaporator (Jyoti scientific, Gwalior), freeze dried and stored in refrigerator until used.

Preliminary Phytochemical Tests

The crude extracts of *Azadirachta indica* was subjected to qualitative tests for identification of different constituents like alkaloids, flavonoids, terpenoids, phenolics, glycosides, saponins and tannins by using standard qualitative methods described by Trease and Evans[4].

Chemical

Streptozotocin (STZ) was purchased from Sigma chemical company. All other chemicals used in the experiments were purchased locally and were of analytical grade.

Animals

Wistar albino rats (150-200 g) of either sex were selected. The animals were housed in polypropylene cages under standard conditions (25±5% °C, 12 h light and dark cycle) with free access to standard pellet feed and water ad libitum. All the experimental procedures and protocols were approved by the Institutional Animals Ethics Committee (Proposal no. IPS/COP/IAEC/04, dated 30/09/2014) as per CPCSEA guidelines. The animals were used for experimentation after one week of acclimatization period.

Preliminary Pharmacological Screening of Extracts

Excision wound model was selected to access the wound healing capability of plant extracts. Semisolid ointment formulations were prepared of all successive extracts. The animals were divided into five groups

(n = 6) and treated topically twice a day.

Group I: Control treated with simple ointment base

Group II: standard treated with 0.005% Fluticasone propionate

Group III: Treated with chloroform extract ointment

Group IV: Treated with methanol extract ointment

Group V: Treated with pet ether extract ointment

Formulation of ointments

Ointment base was prepared by levigation method using prescribed amount of wool fat, cetostearyl alcohol, hard paraffin and yellow soft paraffin**.** This served as control base and dried Methanolic *Azadirachta indica* (MAI) extracts were added to the ointment base to obtain 2.5%(w/w) (F1) and 5% (w/w) (F2) concentration. Fluticasone propionate (Glaxo SmithKline, India) ointment was used as standard for positive control.

Diabetes induction

Diabetes was induced in overnight fasted rats by intraperitoneal injection of freshly prepared solution of streptozotocin (50 mg/kg body weight) in 0.1 M citrate buffer (pH 4.5). Wounds were made on the rats showing elevated blood glucose level (250mg/dl).

Wound healing study

Excision wound model

The animals were divided into eight groups (n = 6) and treated topically once a day, starting from wound induction until complete healing.

Group 1: Non-Diabetic control (treated with ointment base)

Group 2: Non-Diabetic Standard (treated with Fluticasone propionate (0.005% w/w))

Group 3: Diabetic control (treated with ointment base)

Group 4: Diabetic Standard (treated with Fluticasone propionate (0.005% w/w))

Group 5: Non-Diabetic wounds treated with 2.5% w/w methanol extract ointment

Group 6: Non-Diabetic wounds treated with 5% w/w methanol extract ointment

Group 7: Diabetic wounds treated with 2.5% w/w methanol extract ointment

Group 8: Diabetic wounds treated with 5% w/w methanol extract ointment

Streptozotocin induced diabetic wistar albino rats of either sex were anesthetized with intraperitoneal injection of ketamine (50 mg/kg body weight) and their dorsal surface was shaved with a sterile blade. The shaved area was disinfected with 70% (v/v) ethanol. One full thickness circular excision wound (14 mm diameter) was created on the dorsal middle line. Wound contraction was measured as percent contraction in each two days after wound formation[5].

Dead space wound model

This model was used for the study of granuloma tissue. The animals were grouped as mentioned in excision modeland anaesthetized by light ether and wound was made by implantation of polypropylene tube (2.0 x 0.5), in the lumber region on the dorsal surface in each animal. On the 10^{th} post-wounding day, granuloma tissue formed on implanted tube was dissected out carefully. Granuloma tissue was used for determination of tensile strength[6-7].

Statistical analysis

All the results are expressed as mean standard error of mean (SEM). Statistical analysis was performed using one-way ANOVA followed by student t-test. Data were considered significant at $P< 0.05$.

RESULTS

Phytochemical analysis

The phytochemical analysis of crude extracts of *Azadirachta indica* leaves revealed the presence of coumarins, glycosides, tannins, alkaloids, saponins and flavonoids. The presences of these phytochemicals are considered to be responsible for wound healing activity in diabetes (Table 1).

Table 1: Phytochemical screening of leaf extracts of *Azadirachta indica*

S.No.	Test for Plant Constituents	Petroleum Ether	Chloroform	Methanol
1.	Alkaloids	-	-	+
2.	Saponins	+	-	+
3.	carboxylic acids	-	-	-
4.	Flavanoides	+	+	+
5.	Resins	-	-	-
6.	Volatile oils	+	+	+
7.	Coumarins	+	+	+
8.	Xantho proteins	-	-	-
9.	Glycosides	+	+	+
10.	Tannins	+	+	+
11.	Fatty acids	+	+	+

Preliminary Pharmacological Screening of Extracts

Table 2: Percentage wound contraction by different extracts of *Azadirachta indica* in excision wound model in rats.

Days	Simple ointment (control)	Standard ointment (0.005% Fluticasone propionate)	Chloroform extract ointment	Methanol extract ointment	Pet ether extract ointment
4	39.22	28.65	54.68	50.67	33.46
6	42.36	71.35	55.73	66.35	55.15
8	53.12	85.06	68.35	78.81	60.31
10	62.33	92.95	74.56	86.55	67.76
12	69.45	98.52	82.17	94.18	73.53
14	78.62	100	87.46	98.33	81.27
16	85.18		93.37	100	86.35
18	89.33		95.68		97.61
20	96.98		98.44		98.31
22	97.15		100		99.91
24	99.15				100
26	100				

All the extracts of *Azadirachta indica* showed a significant ($P < 0.01$) decrease in wound contraction when compared to control group. Methanol extract required less days (16) for complete wound healing. The standard drug treated animals showed significantly greater wound closure as compared to the extract treated animals. The results demonstrate (Table 2) potential wound healing property of the methanol extract. Further studies may be carried out on methanol extract in order to find out the various wound healing parameter in diabetic and non diabetic rats.

Excision wound model

It was observed that the methanol extracts in both the concentrations of 2.5% w/w and 5% w/w significantly ($P < 0.01$) reduced the epithelization time of animals. In diabetic animals, methanolic extracts showed significantly greater wound healing as comparative to diabetic control animals. Also in normal animals, the methanol extracts showed significantly greater wound healing when compared with normal control animals. The Calculation of wound contraction standard drug-treated animals in both normal and diabetic animals was showed significantly greater wound closure when compared with control and extract-treated animals (Table 3 & 4).

Dead space wound model

Wound parameters

In diabetic animals and normal animals, the methanol extracts in both the concentrations of 2.5% w/w and 5% w/w showed significantly higher levels of hydroxyproline when compared with control animals group. A significant increase was also observed in the dry and wet weight of the granulation tissue in the methanol extracts in both the concentrations of 2.5% w/w and 5% w/w when compares with control animals groups (Table 5 & 6). In diabetic animals, the methanol extracts in both the concentrations of 2.5% w/w and 5% w/w showed significantly greater tensile strength when compared with diabetic control animals (Table 7).

Table 3: Effect of extract and standard ointment on various wound parameters of Excision wound model in non-diabetic rats

Groups	Control/ Formulation	Hydroxyproline (mg/ g tissue)	Protein content (mg/g tissue)	Tensile strength (g/mm2)	Epithelization period (days)
I	Control (Non- Diabetic)	35.25	51.67	3.87	25
II	Std. (Non- Diabetic)	64.85	71.68	5.24	17
V	F1	58.23	64.32	4.95	20
VI	F2	62.29	68.98	5.10	19

Table 4: Effect of extract and standard ointment on various wound parameters of Excision wound model in diabetic rats

Groups	Control/ Formulation	Hydroxyproline (mg/ g tissue)	Protein content (mg/g tissue)	Tensile strength (g/mm2)	Epithelization period (days)
III	Control (Diabetic)	22.94	43.55	1.96	29
IV	Std. (Diabetic)	41.27	62.56	4.28	20
VII	F1	31.26	56.23	3.70	26
VIII	F2	38.29	60.26	3.98	22

Table 5: Effect of extract and standard ointment on various wound parameters of dead space wound model in non-diabetic rats

Groups	Control/ Formulation	Hydroxyproline (mg/ g tissue)	Protein content (mg/g tissue)	Tensile strength (g/mm2)	Granuloma dry weight (mg)
I	Control (Non-Diabetic)	28.17	24.73	2.55	20.75
II	Std. (Non- Diabetic)	61.37	59.12	4.15	58.21
V	F1	41.65	38.27	3.15	51.27
VI	F2	60.59	57.11	3.95	57.25

Table 6: Effect of extract and standard ointment on various wound parameters of dead space wound model in diabetic rats

Groups	Formulation	Hydroxyproline (mg/ g tissue)	Protein content (mg/g tissue)	Tensile strength (g/mm2)	Granuloma dry weight (mg)
III	Control (Diabetic)	15.27	16.68	1.24	13.56
IV	Std. (Diabetic)	51.36	42.81	3.13	57.43
VII	F1	37.15	31.55	2.64	47.82
VIII	F2	42.73	41.68	2.85	56.11

Table 7: Effect of prepared formulations and reference ointment on percent wound contraction on excision wound model in non-diabetic and diabetic rats.

	Non-Diabetic				Diabetic			
Days	Control (Non-Diabetic)	Std. (Non-Diabetic)	F1	F2	Control (Diabetic)	Std. (Diabetic)	F1	F2
4	28.67	54.68	50.56	52.67	15.32	50.71	44.15	45.85
6	42.39	71.35	66.27	67.52	31.59	64.60	62.51	63.14
8	53.16	85.06	78.76	80.53	44.36	80.58	77.64	78.95
10	62.37	92.95	86.52	87.74	55.29	91.74	85.39	86.60
12	69.50	98.52	94.17	96.26	63.74	96.93	92.28	93.99

DISCUSSION

Wound represents a major health problem, both in terms of morbidity and mortality.Diabetes is associated with delays in wound healing, which may be due to the elevation of blood glucose levels, denaturation of proteins and cellular components, overproduction of oxidative free radicals, and alterations in connective tissue metabolism, that is, loss of collagen[8].Wound healing is characterized by three stages, viz., inflammation, proliferation, and remodeling. The proliferative phase typically demonstrates angiogenesis, collagen deposition, granulation tissue formation, epithelialization and wound contraction. Collagen is an extracellular protein in the granulation tissue of a healing wound and acts as a homeostatic agent as well as an initiator of epithelization by providing strength

and integrity to a tissue matrix. These alterations of collagen may be due to decreased levels of synthesis or enhanced catabolism of newly synthesized collagen, or both[9].

The topical application of prepared ointments of MAI improved the breaking strength, wound contraction, and period of epithelization in the excision and dead space models of experimental wounds. The faster wound contraction rate may be due to the stimulation of interleukin or induction of more rapid maturation of granulation tissue. A significant increase in collagen content due to the enhanced migration of fibroblasts and epithelial cells to the wound site was observed during the wound-healing process in the treated group. A close observation of granulation tissue sections revealed that tissue regeneration was much quicker in the treated group compared to diabetic control.

The increase in dry granulation tissue weight in the treated groups indicated high protein content. Collagen is composed of the amino acid hydroxyproline, which has been used as a biochemical marker for tissue collagen[10]. In the dead space wound model, the increase of dry tissue weight and hydroxyproline content was observed in each treatment derived from MAI, which suggests greater deposition of collagen. The formation and maturation of collagen may be due to the presence of flavonoids and steroids in MAI, which are responsible for free radical-scavenging activities and help to promote the most important phase of wound healing. Hence, on the basis of results obtained and above facts, we can say that the faster wound healing activity of MAI in diabetic and normal animals may be due to the presence of phytochemicals and their effect on components of wound healing.

CONCLUSION

The present study concluded that the topical application of methanol extract of *A. indica* leaves applied topically promotes the healing of wounds, with enhanced rates of collagen turnover and wound contraction in diabetic-induced rats. The results also show that application of methanol extract of *A. indica* leaves significantly increased dry and wet weight and hydroxyproline content in both normal and diabetic animals.

On the basis of above, it may be conclude that plant extract promotes wound healing in both diabetic and normal animals by topical application of extracts. This supports its prevalent use in treatment of diabetes and infection.However, further phytochemical studies are needed to isolate the active compounds responsible for these pharmacological activities and will be helpful in projecting this plant as a therapeutic target for healing wounds and treating various diseases.

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