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Healing potential of ethyl acetate soluble fraction of ethanolic extract of *Terminalia chebula* on experimental cutaneous wounds in streptozotocin induced diabetic rats

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Abstract

Introduction: Diabetes is a chronic disorder generated by poor glycemic control; leads to developed several complications including delayed wound healing after any injury. These non healing wound leads to organ or limb salvage. The available modern treatments are not capable to complete control on these complications. There are several examples said that these complications can easily treated by herbal or folklore medicines. The fruit of *Terminalia chebula* used by traditional peoples in the treatment of diabetes and associated wound healing complications. In our previous study we had found that the ethaonilc extract of fruit of *Terminalia chebula* is most active in treatment of wound healing in diabetic rats. The aim of our study was to find the active fraction from ethanolic extract of fruit of *Terminalia chebula,* which is responsible for wound healing activity in diabetic rats.

Methods: The wistar albino rats were made diabetic by single i.p. injection of streptozotocin (60 mg/kg). The excision, incision and dead space wound were implicated on back side of rats. The ethyl acetate soluble fraction of ethanolic extract of fruit of *Terminalia chebula* was applied topically in excision wound model while in incision and dead space wound model the ethyl acetate soluble fraction (100 mg/kg) was give orally for 16 days.

Results: In the excision wound model the wound area and day of epithelization both were significantly decreased with faster wound closure in Ethyl acetate soluble fraction treated rats. There were significant increase in weight of wet & dry granulation tissue with increased amount of hydroxyproline, collagen and elastin was observed in treated rats by ethyl acetate soluble fraction of fruit of *Terminalia chebula* in dead space wound model. In incision wound model significantly higher tensile strength and decreased level of glycated hemoglobin was measured with decrease in blood glucose level in rats treaded with ethyl acetate soluble fraction of fruit of *Terminalia chebula* orally.

Conclusion: The results suggested that the ethyl acetate soluble fraction of ethanolic extract of fruits of *Terminalia chebula* having healing potential in streptozotocin induced diabetic rats.

Keywords: *Terminalia chebula*, Collagen, Diabetes, Granulation, Hydroxyproline, Tensile strength, wound healing.

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1. INTRODUCTION:

Chronic wounds are major problem in large proportion in from of the world. In human population millions of money has expended for the treatment of problems like wound healing.[1] Diabetes mellitus is a disorder provoked by chronic hyperglycemia and generated many complications like foot ulcers and poor wound healing.[2] Diabetes is associated with glycation of essential proteins and enzymes. The glycation process leads to formation of glycated proteins, which are abnormal or defected proteins and reduces the normal functioning of body. Diabetes can generate many tissue abnormalities including connective tissue abnormality. In diabetic patient decrease in collagen content of skin can generates impaired and non healing defects at wound site [3]. Diabetic wounds are slow, non-healing wound that can persist for weeks despite adequate and appropriate care. Such wounds are difficult and tough to manage. The wound healing process is the sequence of repairment of connective tissue including migration, inflammation, proliferation and differenetiation of cells [4]. Animal models are useful tool to study pathophysiological changes in diabetes. The streptozotocin destroyed the β-cell of pancreas and generates hyperglycemia due to lake of insulin. [5]. *Terminalia chebula* is called as mother of herbs and used in treatment of various diseases including diabetes, immunity and wound problems. Ethanolic extract of *Terminalia chebula* contains many phytoconstituents including terpenoids, phenolics & tannins [6]; which are having antioxidant [7], antidiabetic [7, 8], antimicrobial [9] , and immunomodulatory[10] properties. On fractionization and phytochemical screening ethyl acetate fraction of *Terminalia chebula* showed presence of high amount of phenolics & tannins. The tannins are reported to have potant antioxidant [11] and wound healing activity. The oxidative stress is responsible for induction of diabetic complications. Hence in present study ethyl acetate soluble fraction of ethanolic extract of *Terminalia chebula* fruits was used to investigate wound healing activity in diabetic rats.

2. MATERIALS AND METHODS:

Plant Material: The fruits of *Terminalia chebula* were purchased from herbal drug supplier of Mandsaur (M.P.) and authenticated in Department of Pharmacognosy at B. R. Nahata College of Pharmacy-SIRO, Mandsaur (M.P.) India.

Preperation of extract and fraction: Dried fruits of *Terminalia chebula* were extarced with ethanol by successive solvent extraction technique by using soxhlet apparatus for 72 hrs. The ethanolic extract was dried and suspended in water and fractionized with ethylacetate and dried under vaccume and stored in glass container for further use.

Animals: Wistar albino rats of either sex weighed between 120-150 gm were used for the wound healing activity. The animals were housed in central animal house faclity of B. R. Nahata College of Pharmacy-SIRO at controlled standard housing conditions of CPCSEA for temperartue, water and feed. All experimental protocols were approved by Institutional animal ethical committee (IAEC) of B. R. Nahata College of Pharmacy-SIRO, Mandsaur (M.P.) India under proposal number.

Induction of Diabetes: Rats were made diabetic by a single injection of Streptozotocin (60 mg/kg, i.p.) prepared in citrate buffer (0.1 M, pH 4.5) after overnight fasting[6]. Blood was drawn from the tail vein 24 h after the injection and the glucose level was estimated by glucose oxidase method by using Accu-Chek Glucometer befor and 72 hrs after STZ injection. Animals showed blood glucose level more than 250 mg/dl were selected for further cutaneous wound healing activity in diabetic animals [12].

Preparation of ointment of fractions: The ethyl acetate fraction of ethanolic extracts (10 % w/w) of the dried fruits of *Terminalia chebula* triturated in pistal mortar with steric acid ointment base and used further in excision cutaneous wound healing model in diabetic rats.

Excision wound healing model in diabetic rats: Animals were anaesthetized with slight vapour inhalation of di-ethyl ether and the back side of each rat was shaved. Excision wounds sized 300 mm^2 and 2 mm depth were made by cutting out piece of skin from the shaven area. The entire wound was left open. Animals were closely observed for any infection and those which showed any sign of infection were separated, excluded from study and replaced. Wound areas were measured on days 0, 4, 8 and 16 for all groups, using a transparency sheet and a permanent marker. Recording of wound areas were measured on graph paper. The day of scar falling, after wounding without any residual raw wound was considered as the day of epitheliazation [13].

Treatment Groups: For excision wound model:

1. Group I (NC): Normal Control; Normal rats topically treated with Plane steric acid ointment.
2. Group II (DC): Diabetic Control; Diabetic rats topically treated with Plane steric acid ointment.
3. Group III (DT): Diabetes Treated; Diabetic rats topically treated with ointment of ethyl acetate soluble fraction of ethanolic extract of fruits of *Terminalia chebula* (100 mg/kg).

Incision wound healing activity in diabetic rats: Animals were anaesthetized with slight vapour inhalation of di-ethyl ether and the back side of each rat was shaved. A longitudinal paravertebral incision of six centimeters in length was made through the skin and cutaneous muscle on the back in anesthetized rats. After the incision, surgical sutures were applied at intervals of one centimeter. The wounds were left undressed (day 0). The sutures were removed on the 8th post wound day and the application of extract was continued. The skin-breaking strength was measured on the 11th day by tensiometer[14] and glycated hemoglobin level was measured by method of Balamugan R. [15], while blood glucose level was measured by using Accu chek Glucometer based on glucose oxidase method.

Treatment Groups: incision wound model:

1. Group I (NC): Normal Control; Normal rats treated with plane vehicle of 0.5 % w/v sodium CMC orally.
2. Group II (DC): Diabetic Control; Diabetic rats treated with plane vehicle of 0.5 % w/v sodium CMC orally.
3. Group III (DT): Diabetes Treated; Diabetic rats treated with 100 mg/kg of ethyl acetate soluble fraction of ethanolic extract of dried fruits of

Terminalia chebula suspended in 0.5 % w/v sodium CMC suspension orally.

Dead space wound healing activity in diabetic rats: Animals were anaesthetized with slight vapour inhalation of di-ethyl ether and the back side of each rat was shaved. Dead space wounds were inflicted by implanting sterile cotton pellets (10 mg each), one on left side in the groin and axilla on the ventral surface of each rat. On the 11th post-wounding day, the granulation tissue formed on the implanted cotton pellets was carefully removed under anesthesia. After noting the weight of the granulation tissue, the tissue was dried at 60ºC for 12 hr, and the dry granulation tissue weight was recorded [16]. This dried tissue was further used to estimate hydroxyproline [17], collagen [18] and elastin [19] level in skin of normal and diabetic rats.

Treatment Groups: For Dead space wound model:

1. Group I (NC): Normal Control; Normal rats treated with plane vehicle of 0.5 % w/v sodium CMC orally.
2. Group II (DC): Diabetic Control; Diabetic rats treated with plane vehicle of 0.5 % w/v sodium CMC orally.
3. Group III (DT): Diabetic rats treated with 100 mg/kg of ethyl acetate soluble fraction of ethanolic extract of dried fruits of *Terminalia chebula* suspended in 0.5 % w/v sodium CMC suspension orally.

Biochemical analysis: At the end of experiments the wound area, % wound closure and day of epithelization was recorded in excision wound model [13]. In dead space wound model the weight of wet & dry granulation tissue [15], amount of hydroxy- proline [17], collagen [18] and elastin [19] were measured. In incision wound model the tensile strength [14], glycated hemoglobin and blood glcose level was measured

Statistical analysis: The data were expressed in Mean±SEM and statistically analyzed by one-way analysis of variance followed by dunnet's test. P<0.05 considered as significant.

3. RESULTS: There was significant increase in wound healing parameters during treatment with ethyl acetate soluble fraction of ethanolic extract of dried fruits of *Terminalia chebula* as compared to control groups of normal and diabetic rats.

S. No.	Groups		Wound Area (mm^2)	%Wound Closure	Day of Epithelization	Tensile Strength (gm/mm^2)	Blood Glucose Level (mg/dl)	Glycated Hemoglobin Level (mg/dl)
1.	Normal (NC)	Control	57.00± 1.461	81.49±0.422	27.67± 0.881	245.5±2.410	76.33±4.248	4.583±0.090
2.	Diabetic (DC)	Control	90.17± 2.469***	70.37± .099***	40.17±1.302***	170.5±2.280***	298.5± 10.65***	11.70± 0.085***
3.	Diabetic (DT)	Treated	8.50±0.428***	97.31±0.130***	16.33±0.421***	326.4±2.768***	71.33±1.585***	4.233±0.133***

Data are expressed as Mean ± SEM and analyzed statistically by One way ANOVA followed by Dunnett's Multiple Comparison Test, using Graph Pad Prism Software trial version. IN Dunnett's Multiple Comparison Test, Group DC was compared with NC and diabetic treated groups were compared with DC. P value considered as P<0.05 Significant (*), P<0.01 Very Significant (**), P<0.001 Highly Significant (***).

Table No. 1: Effect of ethyl acetate fraction treatment in excision and incision wound model

S. No.	Groups		Wet Granulation Tissue Wt. (mg)	Dry Granulation Tissue Wt. (mg)	Hydroxyproline (µg/ml)	% Collagen	% Elastin
1.	Normal (NC)	Control	226.2±2.151	62.83±1.167	5.508±0.128	41.09±0.957	239.1±5.571
2.	Diabetic (DC)	Control	117.8±2.056***	33.67±1.116***	3.665±0.090***	27.34±0.674***	159.1±3.925***
3.	Diabetic (DT)	Treated	351.8±1.851***	112.7±1.358***	9.472±0.114***	70.66±0.853***	411.1±4.965***

Data are expressed as Mean ± SEM and analyzed statistically by One way ANOVA followed by Dunnett's Multiple Comparison Test, using Graph Pad Prism Software trial version. IN Dunnett's Multiple Comparison Test, Group DC was compared with NC and diabetic treated groups were compared with DC. P value considered as P<0.05 Significant (*), P<0.01 Very Significant (**), P<0.001 Highly Significant (***).

Table No. 2: Effect of ethyl acetate fraction treatment in dead space wound model.

Effect on wound parameters of excision and incision wound model:

As shown in Table No. 1, the effect of ethyl acetate soluble fraction of ethanolic extract of *Terminalia chebula* fruits on wound area; % wound closure and day of epithelialization in excision wound model and tensile strength & blood glucose level in incision wound model in diabetic rats was studied. The ethyl acetate fraction treated rats showed significant increase in % wound closure and decrease in wound area on 16th day of treatment. The day of scar falling i.e. epithelization was decreased with decrease in blood glucose level. In incision wound model the tensile strength of ethyl acetate fraction treated rats was found increased along with decrease in glycated hemoglobin & blood glucose level with comparison to diabetic control rats.

Effect on wound parameters of excision and incision wound model:

As shown in Table No. 2, the effect of ethyl acetate soluble fraction of ethanolic extract of *Terminalia chebula* fruits on wet & dry weight of granulation tissue, amount of hydroxyproline, collagen and elastin. In dead space wound model the weight of wet & dry granulation tissue was significantly increased with significant increase in level of hydroxyproline, % collagen and % elastin in the ethyl acetate fraction treated rats with comparison to diabetic control rats.

4. DISCUSSION:

Diabetes has became critical health problem in modern age . The number of patients suffering with diabetes and its complications increasing regularly and reached more than 200 million in this year [20]. Diabetes is a group of disorders characterized by hyperglycemia resulting abnormalities in glucose metabolism [21]. This diabetes can produced many molecular and cellular abnormalities like connective tissue abnormality including loss of tissue integrity, weak tensile strength, and decreased elasticity. In diabetic patient decrease in collagen content of cutaneous layer of skin can generates impaired and non healing abnormalities in wound area [22]. Abnormal cellular functions related to diabetic wounds includes, delayed inflammation, altered repair and regeneration of blood vessels, decreased collagen synthesis, and defective macrophage function. Diabetic wounds are also prone to infections due to altered granulocytic function and cellular chemotaxis [23]. The streptozotocin has been used as diabetogen to produce high level of blood glucose and production of complications of diabetes [12]. This complication mechanism involved oxidative stress in body produces the delayed wound healing [24]. The phytochemicals like terpanoids, phenolics and tannis [6] are potent antioxidants [7, 11] and can alter the oxidative stress in diabetic patient.

In present study photochemical screening showed the presence of high amount of phenolics and tannin compounds in ethyl acetate soluble fraction of ethanolic extract of *Terminalia chebula* fruits. The Phenolics and tannins are the potent antioxidants reported in literature [7, 11]. Investigators also reported antidiabetic activity of ethanolic extract of *Terminalia chebula* fruits. The high blood glucose level is responsible for delayed wound healing and ethyl acetate fraction treated rats showed significant decrease in blood glucose level during wound healing process.

Deep skin wounds in diabetic and non-diabetic cases heal by contraction and granulation tissue formation and re-epithelialization. In excision wound model the ethyl acetate fraction treated group exhibits faster wound contraction and re-epithelialization. The % wound closure was also more in fraction treated rats. Healing of wounds, a fundamental response to tissue injury occurs by a process of connective tissue repair. A fibrous scar is the end product of wound healing process, the pre-dominant constituent of this is collagen. Collagen and other components of the ground substance are synthesized by the highly vascular granulation tissue that is formed within the wound space. Collagen provides strength and integrity to the repaired dermis [25]. In incisional skin-wound models made on the back of db/db mice, delayed repair was characterized by reduced angiogenesis, delayed formation of granulation tissue, decreased collagen content, and low breaking strength [26]. In incision wound model the increased amount of tensile strength was observed in ethyl acetate soluble fraction of ethanolic extact of *Terminalia chebula* fruits.

In the dead space wound model the rats of ethyl acetate fraction treatment group showed increased inflammation, granulation and skin strengthening in the form of increase in wet & dry weight of granulation tissue with elevated level of hydroxyproline, collagen and elastin content. The hydroxylproline is the constitutory amino acid of collagen and elastion and these are responsible for granulation, strengthening, and remodeling during tissue repair process after injury.

5. CONCLUSION:

The ethyl acetate soluble fraction of ethanolic extract of *Terminalia chebula* fruits was evaluated for wound healing activity in diabetic rats. The high blood glucose level is the basic cause of delayed wound healing in patients with diabetes. The treatment of ethyl acetate soluble fraction promotes wound healing by reduction in blood glucose level, rapid contraction of wound area and increased granulation of tissue with elevated tensile strength. This activity could be due to anti diabetic, antioxidant and antimicrobial activities of phytoconstituents like phenolics and tannins which present in ethyl acetate soluble fraction of ethanolic extract of *Terminalia chebula* fruits. Further studies are needed to identify active compound responsible for faster wound healing activity with detailed mechanism of action.

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