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Wound repair and regenerating effect of ethyl acetate soluble fraction of ethanolic extract of *Cinnamomum tamala* leaves in diabetic rats

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ABSTRACT

Diabetes is a chronic hyperglycemic disorder; leads to developed several complications including delayed wound healing after any injury. These non healing wound ends upto organ or limb salvage. The available modern medications are not capable to fully control over these complications. There are several evidences that these complications can easily treated by using herbal of folklore medicines. The leaves of Cinnamomum tamala used by traditional peoples in the treatment of diabetes and associated wound healing. In our previous study we had found that the ethanolic extract of leaves of Cinnamomum tamala 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 C. tamala leaves responsible for wound healing activity in diabetic rats. 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 created on back side of rats. The ethyl acetate soluble fraction of ethanolic extract of leaves of Cinnamomum tamala 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. In the excision wound model the wound area and day of epithelization both were significantly decreased Ethyl acetate soluble fraction treated rats. In incision wound model the significantly higher tensile strength was observed in rats treaded orally with ethyl acetate soluble fraction. 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. The results suggested that the ethyl acetate soluble fraction of ethanolic extract of leaves of Cinnamomum tamala can be beneficial in treatment of wound healing in diabetic rats.

Key words: *Cinnamomum tamala*, Collagen, Diabetes, Granulation, Hydroxyproline, Tensile strength, Wound healing

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INTRODUCTION

Modern world is facing a critical health problem that is diabetes. The number of patients with diabetes and its complications increasing day by day and reached upto 220 million in this year [1]. Diabetes is a group of disorders characterized by hyperglycemia resulting due to abnormalities in glucose metabolism[2]. Diabetes is associated with glycation of essential proteins and hormones, due to presence of high blood sugar level. The advance glycation leads to formation of glycated proteins, which are abnormal functionless proteins and reduces the normal efficacy of body. This 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 abnormalities in wound or injured area [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 repairmenent of connective tissue including migration, inflammation, proliferation and differeneciation of cells [4]. As per WHO the effective treatment of diabetes and its complications can be possible by using herbal or traditional medicines [5]. In our previous study we had found that the ethanolic extract of *Cinnamomum tamala* leaves has beneficial effect in healing of wounds in diabetic rats. The Ethyl acetate fraction of *Cinnamomum tamala leaves* showed presence of tannins and phenolic compounds which are havinh potant antioxidant activity. The oxidative stress is responsible for induction of diabetic complications. Hence in present study ethyl acetate soluble fraction of ethanolic extract of *Cinnamomum tamala* leaves was used to investigate wound repair and regeneration activity in diabetic rats.

MATERIALS AND METHODS

Plant Material: The leaves of *Cinnamomum tamala* 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.

Preparation of extract and fraction: Dried leaves of *Cinnamomum tamala* 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 expermental 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 [6, 7].

Preparation of ointment of fractions: The ethyl acetate fraction of ethanolic extracts (10 % w/w) of the dried leaves of *Cinnamomum tamala* well triturated in pistal mortar with stearic 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[8] and a permanent marker. Recording of wound areas were measured on graph paper and % wound closure was calculated by formula [9]. The day of scar falling, after wounding without any residual raw wound was considered as the day of epitheliazation [10, 11].

Treatment Groups: For excision wound model:
1. Group I (NC): Normal Control; Normal rats topically treated with Plane stearic acid ointment.
2. Group II (DC): Diabetic Control; Diabetic rats topically treated with Plane stearic acid ointment.
3. Group III (DT): Diabetes Treated; Diabetic rats topically treated with ointment of ethyl acetate soluble fraction of ethanolic extract of leaves of *Cinnamomum tamala* (100 mg/kg).

Incision wound healing activity in diabetic rats: Animals were anaesthetized with slight vapor 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[12, 13].

1.1 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 leaves of *Cinnamomum tamala* 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 [14]. This dried tissue was further used to estimate hydroxyproline [15], collagen [16] and elastin [17] 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 (CtPii-EAC): Diabetic rats treated with 100 mg/kg of ethyl acetate soluble fraction of ethanolic extract of dried leaves of *Cinnamomum tamala* 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[7]. In incision wound model the tensile strength was measured [12, 13]. In dead space wound model the weight of wet & dry granulation tissue [14], amount of hydroxy- proline [15], collagen [16] and elastin [17] were measured.

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

RESULTS

There were significant increase in wound healing parameters during treatment with ethyl acetate soluble fraction of ethanolic extract of dried leaves of *Cinnamomum tamala* as compared to control groups of normal and diabetic rats.

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 *Cinnamomum tamala* leaves 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. 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 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 *Cinnamomum tamala* leaves 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.

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

S. No.	Groups	Wound Area (mm^2)	% Wound Closure	Day of Epithelization	Tensile Strength (gm/mm^2)	Blood Glucose Level (mg/dl)
1.	Normal Control (NC)	49.17± 2.120	84.16±0.780	23.33±0.557	232.4±4.676	80.17±2.651
2.	Diabetic Control (DC)	117.5±2.742***	61.76±0.923***	30.50±0.428***	156.1±2.304***	388.8±11.38***
3.	Diabetic Treated (DT)	10.83±1.014***	96.53±0.307***	13.50±0.991***	323.7±1.911***	75.17±2.971***

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.

S. No.	Groups	Wet Granulation Tissue Wt. (mg)	Dry Granulation Tissue Wt. (mg)	Hydroxyproline (µg/ml)	% Collagen	% Elastin
1.	Normal Control (NC)	222.2±3.049	55.33±1.453	5.575±0.080	41.59±0.602	242.0±3.508
2.	Diabetic Control (DC)	113.2±4.400***	34.83±1.167***	3.703±0.100***	27.63±0.746	160.7±4.342
3.	Diabetic Treated (DT)	341.5±3.233***	111.3±1.856***	8.949±0.065***	66.76±0.485	388.4±2.825

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 (***).

DISCUSSION

Modern world is facing a critical health problem that is diabetes. The number of patients with diabetes and its complications increasing day by day and reached up to 220 million in this year [1]. Diabetes is a group of disorders characterized by hyperglycemia resulting due to abnormalities in glucose metabolism [2]. This diabetes can generate many tissue abnormalities including connective tissue abnormality like loss tissue integrity, weak tensile strength, and decreased elasticity. In diabetic patient decrease in collagen content of skin can generates impaired and non healing abnormalities in wound or injured area [3]. Abnormalities related with diabetic wounds include delayed inflammation, altered neovascularization, decreased synthesis of collagen, and defective macrophage function. Diabetic wounds are also prone to infections due to impaired granulocytic function and cellular chemo taxis [18]. The streptozotocin has been used as diabetogen to produce high level of blood glucose and production of complications of diabetes [6]. This complication mechanism involved oxidative stress in body produces the delayed wound healing [19]. The phytochemicals like flavonoids, terpanoids, phenolics and tannis [20] are potent antioxidants and can alter the oxidative stress in diabetic patient [21].

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 *Cinnamomum tamala* leaves. The Phenolics and tannins are the potent antioxidants reported in literature[22] . Sharma et al [23], and Kar et al, [24] reported that Ethanolic extract of *Cinnamomum tamala* leaves exhibits antihyperglycemic activity 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 ethylacetate fraction treated group exhibits faster wound contraction and re-epithelialization [25]. 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 [26]. 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 [27]. In incision wound model the increased amount of tensile strength was observed in ethyl acetate soluble fraction of ethanolic extract of *C. tamala* leaves.

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.

CONCLUSION

The ethyl acetate soluble fraction of ethanolic extract of *Cinnamomum tamala* leaves was evaluated for wound healing activity in diabetic rats. The all four phases (hemostasis, inflammation, gtanulation and remodeling) of wound healing studied by excision, incision and deadspace wound models. The high bllod glucose level is the root cause of delayed wound healing in patients of diabetes. The treatment of ethyl acetate soluble fraction promotes wound healing by decrease in blood glucose level, faster contraction of wound and increased granulation of tissue with increased tensile strength. This action may be due to anti diabetic, antioxidant and antimicrobial activities of phytoconstituents like phenolics and tannins which present in acetate soluble fraction of ethanolic extract of *Cinnamomum tamala* leaves. Further studies are needed to identify active compound responsible for faster wound healing activity with detailed mechanism of action.

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