INFLUENCE OF TINOSPORA CORDIFOLIA ON WOUND HEALING IN DIABETIC WISTAR RATS

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ABSTRACT :
Hyperglycemia, an elevated blood glucose level, and inappropriate insulin release by the pancreas are metabolic diseases that comprise diabetes. Delayed wound healing and frequent urination are some of the symptoms.When blood travels slowly, it becomes more difficult for the body to provide nutrients to wounds, delaying wound healing.There are four interconnected stages to the healing process of a wound: inflammation, proliferation, remodelling, and maturation.Due to the high cost of modern therapeutic treatments for diabetes, the use of medicinal plants has emerged.There are three groups of male wistar rats in this study: those with normal wounds, those with diabetes, and those with diabetes treated with Tinospora paste.Streptozotocin (10 mg) is used to cause diabetes in rats, which is then followed by the creation of a wound and close monitoring of the lesion.Group 1 shows histopathologically confirmed wound healing in 2-3 weeks, Group 2 shows wound healing in roughly 4 weeks, and Group 3 shows wound healing in 2–3 weeks, which is in the middle of the usual range. Therefore, compared to the control and normal groups, the experimental group shows much faster wound healing.
Keywords:*Diabetes,Tinospora,Wistar rat,Streptozotocin.*

INTRODUCTION

- When blood glucose levels are consistently high, a disorder known as diabetes develops.Because the majority of the energy we get from eating is stored in our blood as glucose. In addition to harming the liver, kidneys, and β-cells of the pancreas, aberrant insulin releases and high blood sugar levels (hyperglycemia) impact protein, carbohydrate, and lipid metabolism (Baynes, 19921).
- The pancreas secretes the hormone insulin, which facilitates the uptake of glucose from meals by cells for energy production.The loss of beta cells in the pancreas or an inadequate response by the body's cells to the insulin that is generated are the two main causes of diabetes (Diabetes information sheet, WHO October 2013).
- Urinary frequency, increased hunger, and increased thirst are symptoms.Damage to the eyes, chronic renal disease, and cardiovascular disease are all potential long-term consequences.
- Diabetes is mainly of three types

Type 1 diabetes :
Type 1 diabetes is autoimmune condition.This means immune system attacks and destroys the beta cells in pancreas which produce insulin.The damage is permanent there may be both genetic and envinormental reasons.

Type 2 diabetes:
Type 2 diabetes is insulin resistance which means the body cannot use sufficient insulin.It stimulates pancreas to produce excess insulin until it can no longer keep fuction.Insulin production decreases that leads to excess blood sugar level.There may be both genetic and environmental reasons.Other health factors include being obesity and lack of exercise.

GESTATIONAL DIABETES:
The Gestational diabetes is caused due to hormones related to insulin blocking produced during the period of pregnancy , this type of diabetis only occurs during pregnancy.

This commonly leads to delayed wound healing in diabetic people.

WOUND:

- Wound is one of the types of injury i.e., skin breakdown,cut or any other body part breakdown.And also puctures in skin,scratches caused by some accidents or by heavy forces etc.,(American Academy of pediatrics(2011),First Aid for families Jones &Barlett).
- It specifically refers to a sharp injury which damages the epidermis of the skin and loss of continuity of epithelium.(leaper dj and harding kg. (1998) wounds: biology and management. oxford university press).

WOUND HEALING:

- Wound healing is function of organisms substitution of injured or damaged tissue by freshly produced tissue and restoration if tissue architecture(Kumar *et al*., 2005).
- Normal wound healing is a complex occurance covering four overlapping phases i.e, inflammation,proliferation,remodeling and maturation(Sumitra *et al*., 2005).
- Before the damage of skin the epidermis & dermis protects as barrier system for the outside environment.
- When the barrier is distributed or got damaged some events set into motion to cover or repair the injury or damage(Rieger S , Z hao H , Martin P , Abe K , Lisse TS January 2015).
- This invents include Blood clotting (Hemostasis) , Inflammation , Tissue growth(Cell proliferation) , & Tissue remodeling (Maturation & Cell differentiation).(Enoch,S.Price,2004)

TINOSPORA CORDIFOLIA:

- Tinospora cordifolia is the one which is a climbing shrub which is native to India ,belongs to family Menispermaceae is commonly called as heart-leaved moonseed, guduchi or giloy and in telugu it is known as tippateega.
- Leaves of Tinospora cordifolia are alternate simple and exstipulate which have long petioles up to 15 cm (6 in) long that are pulvinate and roundish, both at the apex and base with the basal one twisted and longer partially and half way around. It is named as **heart-leaved moonseed** by its heart-shaped leaves and its red colored fruit.
- Tinospora has been reported has antidiabetic and antihyperglycemic (Raghunathan and Sharma in 1969;Kar*et al., 2003),*anti-inflammatory(Upadhyay *et al., 2010*),antioxidant(Chopra *et al., 1992),*antistress(Sharma *et al*., 1995).
- Wound healing properties of Tinospora has been also been reported. (Nema et al., 2012).
- The present study is to evaluate wound healing potential of Tinospora in diabetic animals.
- Now a days Guduchi powder is available in ayurvedic stores because of covid-19 as it helps in boosting up immunity.

REVIEW OF LITERATURE:

Diabetes is the disorders of metabolism i.e.,high blood sugar level(Hyperglycemia),abnormal insulin secretions these events effect metabolism that involves protiens,carbohydrates,fats besides damaging liver,kidney and β-cells of pancreas(Baynes,1991).Major symptoms include frequent urination , delayed wound healing. Wound healing is function of organisms substitution of injured or damaged tissue by freshly produced tissue and restoration if tissue architecture(Kumar *et al*., 2005).The experiments and clinical have indicated that local treatment with insulin may improve wound healing in diabetics (Madibally*et al., 2003).* Moreover ,the modern therapatic treatments of diabetes are very expensive .So recently , the use of medicinal plants has been increased.Tinospora cordifoliahas significant importance in traditional ayurvedic medicine used for all the ages in the treatment of several diseases like high fever, jaundice, diabetis, chronic diarrhoea, cancer, bone

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fracture, pain, asthma, skin disease, poisonous insect, snake bite, eye disorders, wound healing (*Parthipan M, Aravindhan V, Rajendran A Anc Sci Life. 2011 Apr*)Tinospora cordifolia plant has the anti diabetic characteristics (Sharma R et al.,2015). Tinospora powder has been used in enhancing the wound healing process.The streroids , flavonoids , tannins,alkaloids, cardiac glycosides are the major phytoconstituents of Tinospora cordifolia and have been reported to play an anti-diabetic role.(*P S, Zinjarde SS, Bhargava SY, Kumar AR BMC Complement Altern Med. 2011 Jan*)

AIM & OBJECTIVES:

AIM:

- To study the rate wound healing process in diabetic induced rats by using Tinospora cordifolia.

OBJECTIVES:

- To compare the wound healing in normal , control and experimental group(Tinospora paste applied).
- To compare histopathological changes seen in normal , control and experimental group(Tinospora paste applied).

MATERIALS & METHODS:

MATERIALS:

Species & Strain	:	Rattus norvegicus (Wistar rat)
Age at Treatment	:	6-8 weeks
Sex	:	Male
Weight of animals	:	200-300gms
Total No.of animals	:	9
No.of groups	:	3
No.of Animals in group	:	3
Drug for inducing diabetes	:	Streptozotocin(12mg)
Storage	:	Room Temperature
Medication	:	Tinospora cordifolia (10 mg)

HISTOPATHOLOGY MATERIALS:

- Fixative solution (usually commercially available as formalin).Phosphate Buffer (pH=6.8).
- Rubber or Gloves.Protective Clothing.Eyeglasses and Mask.Fume Hood.
- Containers with appropriate lids.Labels and Permanent ink.

Fig1:Tinospora cordifolia

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Fig2 :Tinospora cordifolia Powder

ANIMAL IDENTIFICATION :

Fig3: Male Wistar rat.

- Male wistar rats weighing 200-300 gm.
- Animals are identified by unique cage number.
- The animals are marked with permanent animal numbers before the start of test administration and refreshed weekly thereafter.

HOUSING CONDITIONS:

Animal facilities : Standard polycarbonate cage with stainless steel mesh top grill having for holdpellet food and drinking water .
Bedding material :Clean,autoclaved paddy husk
Cage size : 365 mm L x 202 mm B x 180 mm H.
DIET : VRK (Vinod Ramakrishna Kulkarni) nutritional solutions rodent feed,will be provided *ad libitum* .
WATER: RO water will be provided ad libitum.
ACCULIMATIZTION: The animals were acclimatized to experimental room conditions for a period of five days prior to treatment and all the animals were observed once daily for clinical signs and twice daily for mortality.Only healthy animals were used for the study.

RANDOMIZATION& ALLOCATION:

One day prior the experiment , all animals were subjected to detailed clinical examination.The selected animals were randomized into three groups as follows Each group consisted of 4 male rats.The mean body weight variation of animals across group was minimal and body weight range did not exceed ±20% of the mean body weight of each animal across the study groups.
Animals taken for experimentation:

GROUP1:Normal wound induced
GROUP2:Control(Diabetes induced + wound induced)
GROUP3:Experimental(Diabetes induced+wound induced+Tinospora paste applied)

INDUCTION OF DIABETIS TO WISTAR RAT:

- Diabetis was induced in healthy wistar albino rats weighing about 200 to 300 gms(group animals i.e.,G2&G3) by using streptozotocin(Nagaraja puranik et al., 2009) at a dose of 60mg/kg (12mg) body.wt.

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- streptozotocin is dissolved in water for injection and administered through intra-peretonial region(IP).The blood glucose level of the rat must be read before and after induction of streptozotocin.
- Before inducing diabetes the blood glucose level of the rat is 103mg/dL and after inducing the blood glucose level is 575mg/dL.
- Diabetis was induced within 48hrs of administration of streptozotocin and the animals with blood glucose levels above 350 mg/dL were taken for conducting the study.

Figure4: Showing The Weight of Streptozotocin

Figure5: Showing Inducing Diabetes Into Male Wistar Rat

EXCISION WOUND PROCEDURE:

- The wound is created on the dorsal region above the tail of the male wistar rat.
- For wound induction the dorsal skin of the wistar rats had been removed by applying with hair removal cream so, that we can see the clear results.
- Animals must be anesthetized with Phenobarbital.
- Mark on the hair removed portion with marker properly so, wound will be perfectly seen.
- Wound is created by using scissors and forceps .This type of wound is considered as open wound.
- To create wound we must remove the upper layer of skin.

Figures showing steps in wound creating process:

FIG 8 : Marking hair removed portion

FIG6:During hair removal

FIG 9 : Circular marking for wound creation

FIG7:After hair removal

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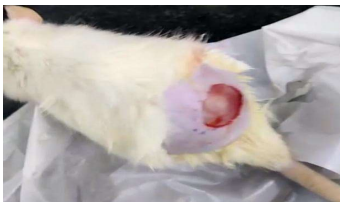
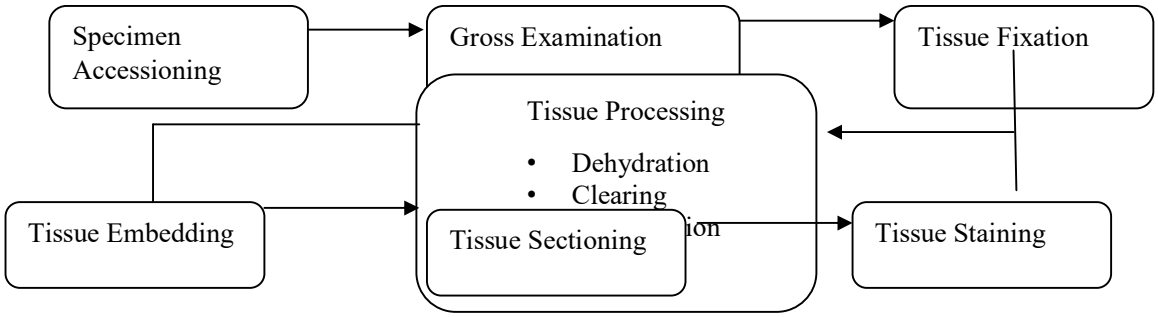


FIG 10: Excision wound created on male wistar

- The Tinospora paste(10mg) is applied on the group III rat wound and kept in observation.
- Then these three groups of animals are kept in the observation for about 21days in room temperature and then tested histopathologically.

HISTOPATHOLOGY PROCEDURE



FIXATION:

Fixation is the preservation of biological tissues from decay due to autolysis.There are several methods of fixation including aldehydes,alcohols,oxidizing agents .Aldehydes is the most frequently used fixation method .

1. Fixative solution (usually commercially available formalin).
2. Phosphate buffer (pH = 6.8).
3. Rubber or gloves.
4. Protective clothing.
5. Eyeglasses and mask.
6. Fume hood.
7. Containers with appropriate lids (volume is commensurate with sample size. Large neck plastic containers are preferable and can be reused).
8. Labels and permanent ink.

METHODOLOGY :

Autolysis is a combination of postmortem changes due to rupture of cell homeostasis that leads to uncontrolled water and electrolytes dynamics in and out of the cell and of alteration of enzymatic activity. These changes are favorable conditions for bacterial and fungal growth and ultimately result in complete destruction of tissue structures. To halt autolysis, tissues should be preserved in an appropriate fixative that permanently cross-link its proteins and stabilize it. The process of autolysis virtually begins immediately after death. Therefore, rapid and adequate fixation after sampling is essential. This can be achieved by immersion of the tissue sample in an adequate volume of fixative solution. There are several methods of fixation including aldehydes, mercurials, alcohols, oxidizing agents, and picric acid derivatives. Tissue immersion in aldehyde (formaldehyde or glutaraldehyde) is the most frequently used fixation method in biomedical research. Formalin (formaldehyde) is

commercially available as 38–40% or 10% neutral phosphatebuffered solutions . It is generally accepted that a volume ratio of tissue to fixative of 1:10 to 1:20 is necessary for optimal fixation. Small tissue samples are usually fixed at room temperature after 12–48 h. Larger specimen may require more fixation time as formalin slowly penetrates tissues.

1. Place the fixative container under a fume hood.
2. Plunge tissue samples in the fixative solution.
3. Stir gently the fixative container for a few seconds to make sure the tissue sample does not stick to the container surface.
4. Replace cap over the container after each tissue (one container can be used for several tissue samples).
5. Add identification label on each container.

Fig11:Tissue Processor

TRIMMING:

1. Fume hood. Rubber or gloves . Protective clothing. Eyeglasses and mask.Dissecting board (plastic boards are preferred as they can be easily cleaned and autoclaved).
2. Blunt ended forceps (serrated forceps may damage small animal tissues).
3. Scalpels blades and handle.
4. Plastic bags and paper towels.
5. Containers for histological specimens, cassettes and permanent labels. Containers and cassettes, should be correctly labelled before starting tissue trimming.

After fixation, tissue samples need to be properly trimmed to reach the adequate size and orientation of the tissue. This step is also important to reach a sample size that is compatible with subsequent histology procedures such as embedding and sectioning. Hard tissues (such as bones and teeth) must be decalcified before trimming.

1. Under a fume hood, remove tissue samples from the fixative container or jar.
2. Trim one or more small pieces of tissues and organs and fit them into cassettes.
3. Place a lid on the cassette.
4. Label each cassette with a permanent ink.
5. Store cassettes in a fixative container.

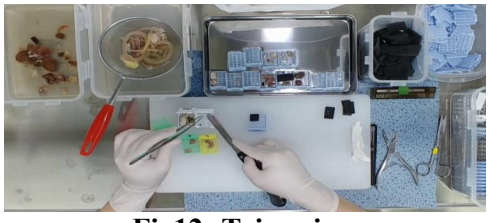


Fig12: Trimming

PRE-EMBEDDING:

1. Disposable plastic cassettes for histology (with appropriate lids). For small samples, disposable plastic cassettes for histology with subdivision (Microsette).
2. Foam pads (31 × 25 × 3 mm) can be used to immobilize tissue samples inside the cassettes.
3. Commercial absolute ethyl alcohol and 96% ethanol solution. 4. 90% and 70% ethanol solutions.
4. Paraffin solvent/clearing agent: xylene or substitute (e.g., Histosol, Neoclear).
5. Histology stainless steel embedding molds. These are available in different sizes (10 × 10 × 5 mm; 15 × 15 × 5 mm; 24 × 24 × 5 mm; 24 × 30 × 5 mm, etc.). 4. Small forceps.

METHODOLOGY :

The goal of pre-embedding is to infiltrate tissue samples with paraffin and replace water content of tissue by this wax material . Paraffin is used as a supporting material before sectioning. Histology grade paraffin wax has a melting point around 56 or 57°C, a temperature that does not alter the structures and key morphologic characteristics of tissues, thus allowing adequate microscopic evaluation by the pathologist. At room temperature, paraffin wax offers enough rigidity to allow very thin sections just a few micrometers thick (usually 4 or 5 Mm). Pre-embedding is a sequential process that consists of dehydration of tissues in increased concentrations of alcohol solutions, then gradual replacement of alcohol by a paraffin solvent. Xylene (or its substitutes; e.g., Histosol, Neoclear, and Histoclear) has the advantage to be miscible in both alcohol and paraffin. As a result, the tissue sample is dehydrated and fully infiltrated by paraffin. This step is generally automated using a variety of vacuum or carousel type tissue processors. When using a tissue processor, the following steps should be followed:

1. Check if the baskets and metal cassettes are clean and free of wax.
2. Do not pack the tissues too tightly to allow fluid exchange.
3. Check if the processor is free of spilt fluids and wax.
4. Check if the fluids levels are higher than the specimen containers. Select the appropriate protocol and check the clock.
5. Infiltrate with paraffin for 1 h (two times).
6. Tissue sampled are retrieved at the end of the processing program (automates are usually run overnight to start the embedding process in the next morning). The following is a list of rescue procedures that can be helpful to consider in case the pre-embedding procedure is not completed normally: 1. Recovery of tissues that have air-dried because of mechanical or electrical failure of the processor:
    (a) Rehydrate the tissue in Sandison's solution (absolute alcohol 30 mL, formaldehyde 37% 0.5 mL, and sodium carbonate 0.2 g water up to 100 mL) or Van Cleve and Ross' solution (trisodium phosphate 0.25 g in 100 mL of water).
    (b) Immerse the tissues in one of these two solutions for 24–72 h (actually most of tissues rehydrate and soften within 4–6 h).
    (c) Process to dehydration and pre-embedding as usual, starting in 70% ethanol.

Recovery of tissues accidentally returned to fixative following wax infiltration. Discard all contaminated fluids:

- Rinse in 70% ethanol followed by 95% ethanol.
- Rinse in absolute ethanol (two to three times).
- Rinse in xylene (or substitute).
- Carry out the paraffin infiltration three times, 30–60 min each.

Recovery of tissues accidentally returned to ethanol 70% following wax infiltration:

- Discard all contaminated fluids.
- Rinse in ethanol 95%.

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- Rinse in absolute ethanol (two changes).
- Rinse in xylene (or substitute).
- Carry out the paraffin infiltration two to three times, 30–60 min each. The same steps can be used for manual tissue processing. Melt the paraffin in an oven at 60°C in glass containers. Immerse the specimens into the melted paraffin.

SECTIONING :

1. Rotary microtome.
2. Tissue water bath with a thermometer. Alternatively a thermostatic warm plate can be used.
3. Disposable microtome blades (for routine paraffin sections use wedge-shaped blades).
4. Sharps container to discard used blades.
5. 0.1% gelatin in water (1 g of gelatin in 1 L of distillated water). This should not be used with Superfrost or Superfrost Plus slides and should be reserved for immunohistology sections. \

METHODOLOGY

Once tissue samples are infiltrated by paraffin, they are removed from the cassettes and carefully positioned inside a metal base mold. This step is critical as correct orientation of the tissue is essential for accurate microscopic evaluation. The mold is filled with melted paraffin and then immediately placed on a cooling surface. To trace each tissue specimen, the cassette with permanent tissue and study identification is placed on top of the metal base mold and incorporated in the paraffin block before cooling. In this manner, the cassette will be used as a base of the paraffin block for microtome sectioning (once the metal base mold is removed)

1. Check that the different compartments of the station have the appropriate temperature. Paraffin should be liquid in the paraffin reservoir, work surface should be warm, and cool plate should be cold. Stainless steel molds should be kept warm.
2. Remove the cassettes from the last tissue processor bath (normally melted paraffin) and transfer to the warm compartment of the embedding station.
3. Transfer one cassette onto the hot plate.
4. Snap off the cassette lid and discard it.
5. Snap off the mold.
6. Bring the paraffin blocks together.
7. Store the paraffin blocks at room temperature until sectioning.

Fig13:Sectioning

STAINING :

1. Harris hematoxylin (commercial solution, ready to use).
   Eosin Y solution.
2. Hydrochloric acid 37%.
3. Absolute ethanol.
4. Ethanol 96%.

5. Glass cover slips (25 × 60 mm).
6. Filter paper.
7. Ethanol solutions: (a) Add 12.5 mL of water to 1 L of commercial 96% ethanol to obtain 95% ethanol. (b) Add 408 mL of water to 1 L of commercial 96% ethanol to obtain 70% ethanol.

METHODOLOGY:

Unstained paraffin sections offer very low contrast and therefore cannot be evaluated microscopically in routine histopathology. It is necessary to apply coloring reagents (mostly chemicals) to stain tissue structures. There are many histochemistry staining techniques that can be applied to examine specific tissue or cell structures. As most of these dyes are water soluble, tissue sections should be rehydrated to remove paraffin (using xylene, alcohol solutions ending in water). Hematoxylin and Eosin (H&E) is the routine staining used to study histopathology changes in tissues and organs from animals in toxicity studies. Hematoxylin is a basic dye that has affinity for acid structures of the cell (mostly nucleic acids of the cell nucleus), and eosin is an acidic dye that binds to cytoplasmic structures of the cell. As a result, H&E stains nuclei in blue and cytoplasms in orange-red. A variant to this staining method is the Hematoxylin–Eosin and Saffron (HE&S) stain. As compared to the H&E method, HE&S stains collagen in yellow-orange, allowing a better highlight of interstitial conjunctive tissueThe following protocol describes manual H&E staining technique. It is suitable for small series of slides. This operation is usually automated to allow high-throughput staining of slides.

1. Prepare the Harris hematoxylin working solution. Filter the commercial solution through filter paper to remove the metallic precipitant that forms in the solution upon standing.
2. Prepare 0.1% aqueous eosin Y working solution. Dissolve 1 g of eosin Y in 1 L of deionised water. Add four drops of HCl to obtain a pH between 4 and 5.
3. Add a thymol crystal to prevent molds growth.
4. Label and date the solutions. Aqueous eosin solution is stable for at least 2 months at room temperature.
5. Prepare the differentiation solution (acid alcohol). Add 1 mL of 37% HCl to 100 mL of 70% ethanol.
6. Dewax the paraffin sections in xylene 2 × 5 min each.

The following protocol describes manual cover slip mounting technique. It is suitable for small series of slides:

1. Wipe the surface under the slide while keeping the tissue section covered with the clearing agent.
2. Apply two or three drops of the mounting medium.
3. Place a cover slip on the slide and avoid the formation of bubbles. Press gently with forceps to remove any bubble.
4. Dry the slides overnight at room temperature on a flat surface within the fume hood. To allow high-throughput slide preparation, this operation is usually automated. These automated machines are commercially available from many suppliers.

RESULT:

- In normal group(I) the wound healing process i.e., complete re epithelialization occurs in 2-3 weeks.
- In control group (II) which is wounded and diabetic induced the wound healing process takes longer time for about 4 weeks.
- In the third group the wound is treated with Tinospora paste(10mg), the rate of wound healing i.e., complete re epithelialization occurs in 2-3 weeks equal to normal group.
- The histopathological report also confirm the rate of wound healing in I and III groups.

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Normal group:

0thday	5thday	11thday	15thday	21stday

CONTROL GROUP:

0thday	5thday	11thday	15thday	21stday

HISTOPATHOLOGY REPORT:

Group 1:Normal group

Days	Organ	Histopathological observation
21st day	Skin	Complete re epithelialisation with epidermal,dermal and sub-cutaneous region appeared normal

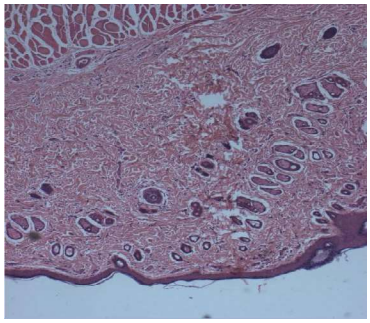

Fig14:Normal Group Histopathological Report

Group 2: Control group

Table 2:

Days	Organ	Histopathological observation
21stday	Skin	Partial re epithelialization with epidermal hyperplasia and moderate inflammation.

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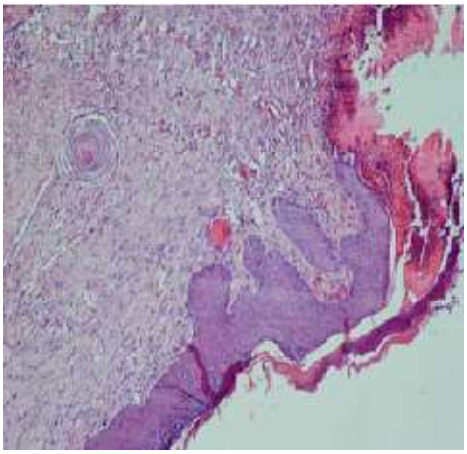

Fig15:Control Group Histopathology Report

Group 3:Experimental group:

Table 3:

Days	Organ	Histopathological observation
21st day	Skin	Complete re epithelialisation with normal epidermal and normal dermal layer of skin.

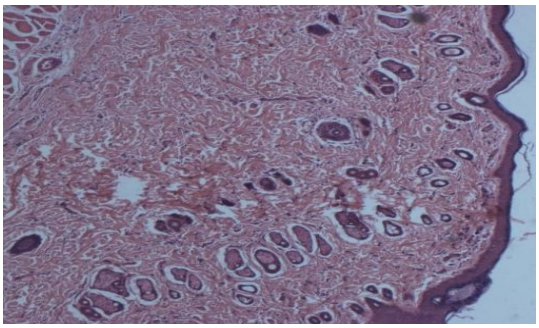

Fig16:Experimental group Histopathology Report

Histopathological Report**:**

Based on histopathological observation and scoring on re-epithelization, inflammation and fibrous tissue proliferation the below clinical significance has obtained in normal group there is complete re epithelialization with epidermal,dermal and subcutaneous region appeared normal in 21days, in control group(diabetic induced and wound induced animals) observed with partial epithelization with severe hyperplasia of epidermal layer along with moderate inflammation was observed in about 4weeks andin experimental group there is complete re epithelization with normal epidermal and normal dermal layer of skin in 21days equal to the normal group .When we compare the therapeutic efficiency of experimental group with normal and control, experimental group showed equal potent wound healing effect with normal group wounds.

DISCUSSION :

- Based on the histpathological observation and scoring on re-epithelialization , inflammation and fibrous tissue proliferation the results are obtained.
- The normal and experimental group have shown complete wound healing i.e.,re-epithelialization with normal and dermal layer of skin in 21 days.

- Control group have shown partial re-epithelialization and delayed wound healing about 4weeks..
- Skin provides a protective barrier that prevents germs and foreign invaders from getting inside the body. When a cut break thebarrier,the immune system is responsible for fighting off and stopping infections.Butin case of diabetes, the body produces enzymes and hormones that make immune system less effective. This can lead to infections that may cause diabetic wounds to take longer to heal and require medical attention.
- As wound healing is delayed in diabetes due to poor circulation, the blood moves slowly which makes more difficult for the body to supply nutrients to wounds, so by using Tinospora paste the rate of wound healing is increased in diabetes.

CONCLUSION :

- In the present study , Tinospora cordifolia have shown better potential for wound healing in diabetes when it is applied as paste.
- When we compare the therapeutic efficiency of experimental group (Tinospora paste) with control and normal group , the experimental group showed more potent wound healing effect in diabetic wounds.
- Hence in the experimental group when the Tinospora paste is applied it has shown equal wound healing with normal group.

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