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Effect of Combination of Some Phytoconstituents of Pharmacological Interest on Cutaneous Wound Healing in Diabetic Rats

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Abstract: Persistent non-healing wounds present a substantial challenge in the context of diabetes management. This research delves into exploring the wound healing capabilities of a combination of natural plant-derived compounds, namely embelin, berberine, ferulic acid, and asiatic acid, in streptozotocin induced diabetic rats. Various wound models, including dead space, incision, and excision models, were employed to evaluate their impact. The phytoconstituents were carefully blended into an ointment formulation, with a concentration of 5% (w/w), specifically designed to cater to their individual strengths. Notably, diabetic rats exhibited a significant enhancement in wound closure and improved skin epithelialization. Furthermore, this combination of phytoconstituents exhibited efficacy when administered orally in both the incision and dead space wound models. In the incision wound model, granulation tissues were examined on the 8th day following the initial injury, and assessments were made for hydroxyproline, hexosamine, total protein. In streptozotocin-induced diabetic rats, both topical and oral applications of this combination revealed a marked increase in hydroxyproline, hexosamine, indicating a favorable impact on wound healing. Additionally, the combination led to a substantial improvement in wound breaking strength. When assessed in the dead space model, the combination notably increased the weight and breaking strength of granuloma tissue. These findings collectively point to the wound healing acceleration attributed to Combination in diabetic rats.

Keywords: Diabetes, wound healing, phytoconstituents, embeline, berberine, ferulic acid Asiatic acid.

Research Paper

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INTRODUCTION

In the realm of wound science, a wound can be described as the disruption or damage to the cellular, anatomical, or functional integrity of living tissue [1]. Wound healing, the intricate process of reestablishing this integrity in living tissue, is a meticulously orchestrated phenomenon. It encompasses a series of interrelated processes, commencing with the initiation of an acute inflammatory response triggered by the injury. This is followed by the rejuvenation of parenchymal cells, the migration and proliferation of both parenchymal and connective tissue cells, the synthesis of extracellular matrix (ECM) proteins, the transformation of connective tissue and parenchymal components, and ultimately, the acquisition of wound strength [2].

The global prevalence of diabetes is on a troubling upward trajectory, with more than 463 million individuals worldwide affected [3]. This issue, deemed an epidemic by the World Health Organization, is predicted to worsen in the near future. One of the most concerning complications of diabetes is impaired wound healing, notably in diabetic foot ulcers, which can lead to severe complications such as infections and amputations [4].

Phytoconstituents, bioactive compounds sourced from botanical origins, possess a rich historical legacy in traditional medical systems due to their diverse pharmacological attributes. A plethora of phytoconstituents has exhibited notable antioxidant, anti-inflammatory, and wound-healing capabilities. The intrinsic advantage of phytoconstituents lies in their

multifaceted mode of action, frequently exerting influence on various targets within biological systems. This multifactorial modus operandi resonates harmoniously with the intricate intricacies associated with impediments in diabetic wound healing, rendering phytoconstituents as compelling subjects for research investigation [5].

Embelin, a naturally occurring hydroxy benzoquinone with alkyl substitution found mainly in Embelia ribes Burm, has demonstrated a wide range of therapeutic properties. These include antidiabetic, antifertility, chemopreventive, antitumor, anti-inflammatory, analgesic, antioxidant, wound healing, and antibacterial activities. Furthermore, embelin has shown protective effects against acetic acid-induced ulcerative colitis in rats. Importantly, it exhibits a high safety margin, with an LD50 of up to 2000 mg/kg body weight in rats and mice, indicating a wide therapeutic index [6, 7]. Berberine is another naturally occurring compound known to possess extremely promising biological and pharmacological activities [8]. Ferulic acid may contribute to diabetic wound healing through its multifaceted mechanisms, including anti-inflammatory and antioxidant effects, promotion of collagen synthesis, angiogenesis stimulation, regulation of matrix metalloproteinases, enhancement of cell proliferation and migration, and potential reduction in scar formation [9]. Asiatic acid is known to enhance diabetic wound healing through its anti-inflammatory, antioxidant, collagen-stimulating, angiogenesis-promoting, MMP-regulating, cell proliferation-boosting, and potential scar-reducing properties [10].

Embelin, berberine, ferulic acid, and asiatic acid each offer a unique spectrum of therapeutic properties, including anti-inflammatory, antioxidant, collagen-promoting, angiogenesis-stimulating, and wound healing effects. By combining these diverse compounds, we aim to capitalize on their collective strengths, potentially enhancing the overall efficacy of wound healing in diabetic individuals. Furthermore, this multi-phytoconstituent approach aligns well with the intricate nature of diabetic wound healing impairments, providing a comprehensive and holistic strategy to address the complex mechanisms involved in this challenging clinical scenario.

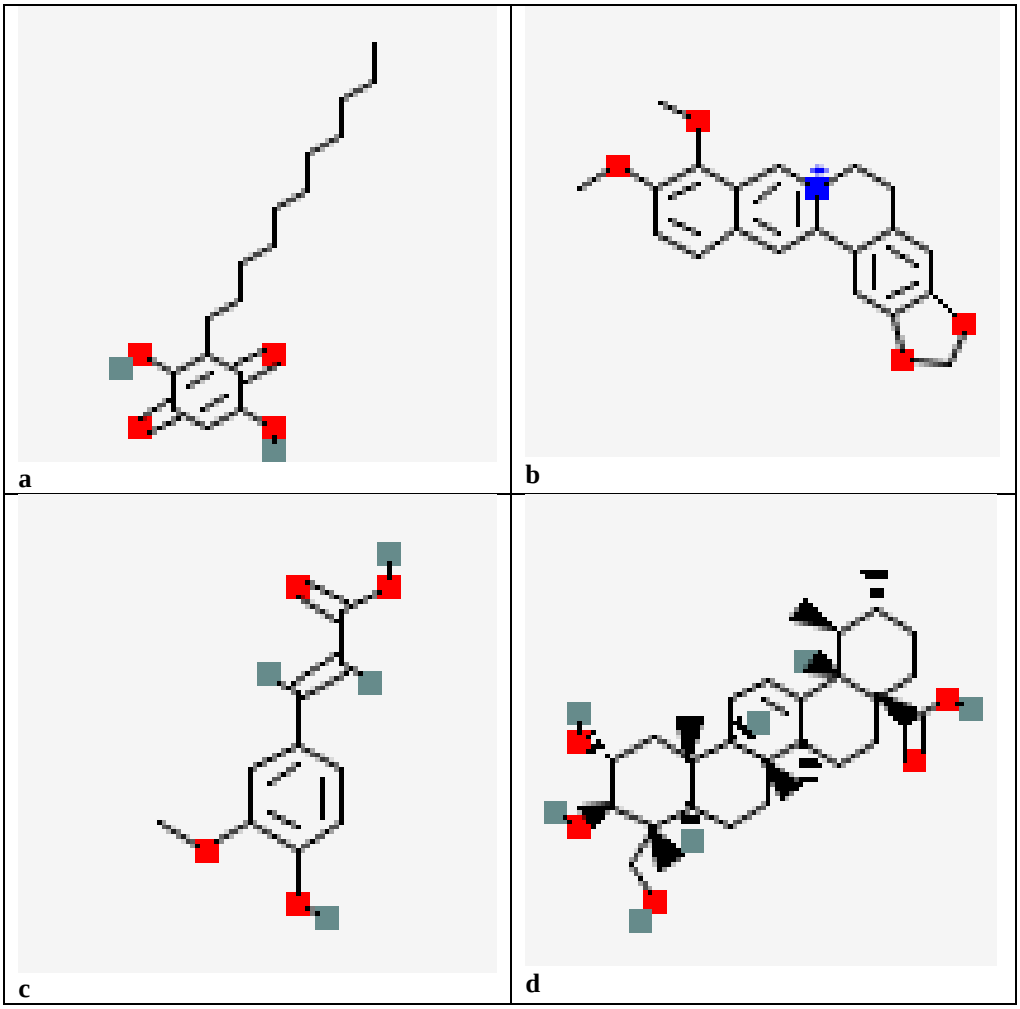

Fig 1: Chemical structure of a) embelin b) berberine c) ferulic acid d) asiatic acid

MATERIALS AND METHODS

Animals:

Wistar albino rats of either sex weighing between 180 and 200 g were obtained from Janata Shikshan Prasarak Mandal's, Sudhakarao Naik institute of Pharmacy, Pusad, Dist, Yavatmal, Maharashtra. The study was approved by the Institutional Ethics Committee for animal experimentation. Rats of wistar strains were used for the study. The rats were maintained in the temperature 22±2℃ and 60-70% relative humidity. The animal were exposed to 12 hrs dark and light cycle, fed *ad libitum* with commercial pellet diet and had free access to water [11].

Induction of diabetes

Diabetes was induced in animals by a single subcutaneous injection of the pancreatic b-cell toxin STZ (Sigma Chemical; freshly dissolved in sterile saline, 0.9%) at a dose of 65 mg/kg body weight. Using glucometer blood glucose levels were monitored 3 days after STZ injection and throughout the duration of study to determine the hyperglycemic state of the animals.

Drug formulations and grouping of animals

A combination was developed in two types of medication formulations for topical and oral administration. Formulation was produced in ointment dosage form utilising simple ointment BP as a foundation for measurement of excision wound healing activities. 2.5 g of combination of embelin, berberine, ferulic acid and asiatic acid depending on their relative strength was combined in 50 g of simple ointment base BP to make ointment with a 5% (w/w) concentration. Combinations (25mg and 50 mg/kg) was chosen from for oral delivery. Animals were divided into three groups for topical treatment (excision and incision wound models): Group I was a diabetic control, Group II was treated topically with a 5% combination ointment containing embelin, berberine, ferulic acid and asiatic acid, and Group III was a diabetic standard treated with povidone iodine ointment. The animals were divided into three groups of six rats for oral administration (incision and dead space wound models). Group I served as diabetic control (vehicle treated), Group II treated with combination (25 mg/kg) orally, and Group III served as diabetic standard treated with combination (50 mg/kg) orally.

Excision wound model

With the use of a circular seal, a standard wound of 2 cm in diameter was created [12]. The percentage wound closure, epithelization time [13] and scar area on complete epithelization were measured.

Parameters used to assess Excision wound healing activity

a) Measurement of surface area of wound

To assess the area of the healing wound, the surface area was measured by tracing the boundary on semitransparent paper and calculation was done using graph paper.

b) Percentage of wound contraction

Wound contraction (WC) was calculated as a percentage change in the initial wound size % of wound contraction (WC) = [(Initial wound size-specific day wound size)/Initial wound size] X 100.

c) Period of Epithelisation and Scar Area

Changes in wound area were measured regularly and the rate of wound contraction calculated as given in the formula below. Significance in wound healing activity of the test groups were derived by comparing treat wound area on respective days with healed wound area of control group. The period of epithelization, i.e. day of fall of eschar and the scar area were also noted down. % wound contraction = Healed Area ×100 Total Wound Area (Healed Area = original wound area – present wound area).

Incision wound model

On either side of the spinal column, two 5 cm long para-vertebral straight incisions were made through the whole thickness of the skin. By sharp scalpel [14]. After complete homeostasis the wound were closed by means of interrupted sutures placed at equidistance points about 1 cm apart. On the 7th day sutures were removed and on the 10th post-wounding day tensile strength was measured by continuous water flow technique [15].

Dead Space wound model

Three groups of six rats each were created from the animals, and each group was housed in a separate cage. The dead space wound was created by implanting subcutaneously polypropylene tubes (2.5 cm × 0.5 cm) in the lumbar region on dorsal side. Animals received combination from 0 day to 9th post-wounding day. On the tenth post-wounding day, each implanted tube harvested granulation tissue was carefully removed with the tube and used to calculate the breaking strength.

Estimation in wet/dry granulation tissue biochemical parameters

On the 18th post-wounding day, the animals were sacrificed and granulation tissue formed on the implanted tubes was carefully dissected out. Tissues were weighed and their weight was expressed as mg/100 g body weight of animal. Tissues were divided into two parts viz. wet tissue (≈220 mg) and dried (>50 mg) granulation tissue for the estimation of various biochemical parameters.

a) Estimations in wet granulation tissue

Wet tissue (220 mg approx. made into 100 mg/ml homogenate) used for the estimation of protein. The granulation tissues were homogenized at 4°C in Phosphate buffered saline [PBS: 1.761 gm KH_2PO_4 + 3.634 gm $Na_2HPO_4.2H_2O$ in 50 ml DW (pH = 7.0)].

a) Protein (Lowry *et al*., 1951)

The protein content of the granulation tissue was estimated using the method of Lowry *et al.*, To 0.1 ml homogenate (equivalent to 10 mg tissue) was added 0.9 ml Absolute alcohol. It was centrifuged at 3000 rpm for 5 minutes. The precipitate so obtained was dissolved in 1 ml of 0.1N NaOH. Out of this 1 ml solution, 0.4 ml was taken into another test tube and to this added 4 ml of alkaline reagent and kept for 10 minutes. Then 0.4 ml of the phenol reagent was added and again 10 minutes were allowed for color development. Readings were taken, against the blank prepared with water, at 610 nm. The protein content was calculated from the standard curve prepared with bovine albumin and expressed in terms of mg/g wet tissue [16].

Estimation in dry granulation tissue

Approximately 250 mg of wet tissue was dried at 50°C for 24 h. Dried tissue was weighed and kept in glass stopper test tubes. Their dried weight was expressed as mg/100 g body weight of animal. It was subsequently used for the estimation of protein and connective tissue determinants like hydroxyproline, hexosamine.

Estimation of connective tissue parameters

To each tube containing 40 mg of the dried granulation tissue, 1 ml of 6N HCl is added. The tubes were kept on boiling water bath for 24 hours (12 hours each day for two days or 8 hours each day for 3 days) for hydrolysis. The hydrolysate was then cooled and excess of acid was neutralized by 10N NaOH using phenolphthalein as indicator. The volume of neutral hydrolysate was diluted to a concentration of 20 mg/ml of dried granulation tissue in the final hydrolysate with distilled water. The hydrolysate was used for the estimation of hydroxyproline, hexosamine following the standard procedures.

Estimation of protein (Lowry *et al*., 1951)

5 mg dry tissue dissolved in 2.5 ml of 0.1N NaOH. To 0.4 ml of hydrolysate was added 4 ml of alkaline reagent and kept for 10 minutes. Then, 0.4 ml of the phenol reagent was added and again 10 minutes were allowed for color development. Readings were taken immediately, against the blank prepared with water, at 610 nm. The protein content was calculated from the standard curve prepared with bovine albumin and expressed in terms of mg/g dry tissue. The protein content of the granulation tissue was estimated using the method of Lowry *et al*., [16].

Estimation of hydroxyproline content:

50 mg of each tissue with 1 ml of 6N HCl were taken in respective screw cap glass vials and autoclaved for 3 hrs at 15 lb pressure to prepare protein hydrolysates. Then the hydrolysates were neutralized to pH 7.0 with 0.1 M NaOH solution. One ml of each hydrolysate was taken in respective test tube to which added 1 ml of 0.01 M CuSO4 solution, 1 ml of 2.5 N NaOH solutions and 1 ml of 6% H2O2 solution. The mixture was allowed to mix for 5 minutes by occasional shaking and kept for 5 minutes on water bath at 800C. Then the tubes were cooled in ice-cold water and added 4 ml of 3N H2SO4 solutions. 2 ml of pdimethylamino benzaldehyde solution was added to the preparation and heated on water bath at 700C for 15 minutes. Similar preparation was made for blank by taking 1 ml of distilled water instead of protein hydrolysate. Then the absorbance was taken at 560nm against reagent blank.

b) Estimation of Hexosamine (Dische and Borenfreund, 1950)

0.05 ml of hydrolyzed fraction (1 mg of dry granulation tissue) was diluted to 0.5 ml with distilled water. To this was added 0.5 ml of acetyl acetone reagent. The mixture was heated in boiling water bath for 20 minutes then cooled under tap water. To this 1.5 ml of 95 % alcohol was added, followed by an addition of 0.5 ml of Ehrlich's reagent. The reaction was allowed for 30 minutes to complete. Color intensity was measured at 530 nm against the blank prepared by using distilled water. Hexosamine contents of the samples were determined from the standard curve prepared by using D (+) glucosamine hydrochloride. The Hexosamine content was expressed as µg/mg protein [17].

The histopathology study of the skin

Histology of newly formed on the wounds was carried out on 18th post wounding day by fixing the skin in 10% formalin. Paraffin sections (5-10µ) were prepared stained with haematoxylin and eosin, and finally mounted in DPX (Di-N-Butyle Phthalate in xylene) medium. Histopathological examination were performed to study the process of epithelisation on the excised wound and to find out evidence of granuloma, dysplasia, oedema and malignancy in the skin under examination [18].

Statistical Analysis of data:

Results are expressed as mean ± SD. The difference between experimental groups was compared using one way Analysis of Variance (ANOVA) the results were considered statistically significant at $p < 0.05$.

RESULTS

Induction of diabetes:

Blood glucose levels were significantly elevated in rats administrated with STZ (332.37±3.21) from. Blood glucose levels for the normal rat control group were (89.23±2.3).

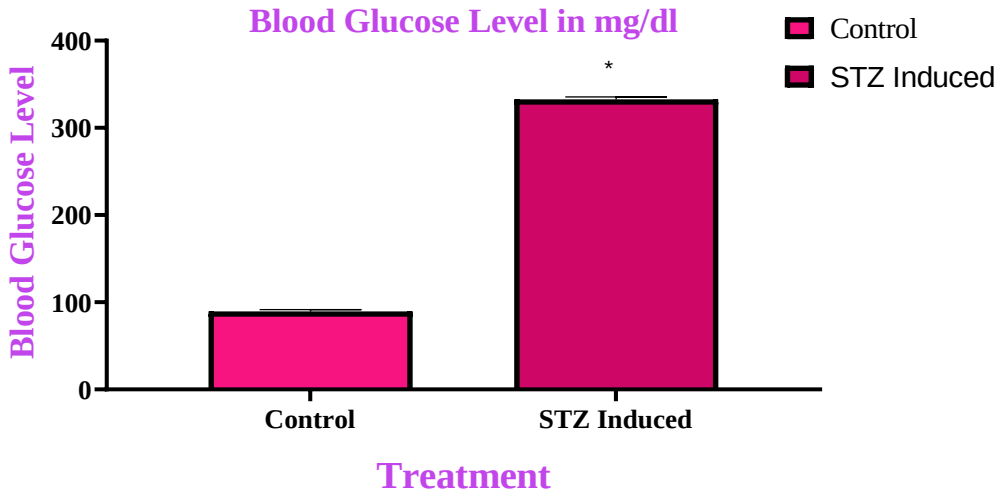

Fig 2: Effect of STZ treatment on blood glucose

The results are depicted in Figure 3. The result indicate that the % HbA1c levels in the STZ Induced group are 11.8 ± 0.4. These results confirms induction of diabetes after administration of STZ in rats.

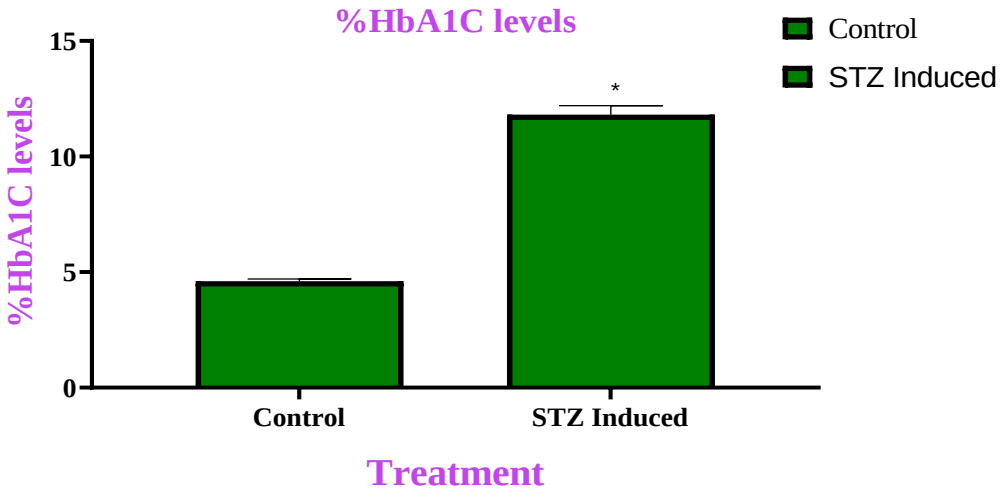

Fig 3: Effect of STZ treatment on % Hb A1C level

Effect of Treatment of Excision wound with Combination on STZ induced diabetic rat

The given results represent the effects of different treatments on wound healing, specifically in terms of wound contraction, epithelization period, and scar area.

Table 2: Effect of Treatment of Excision wound with Combination on STZ induced diabetic rat

Treatment	Percent Wound Contraction					Epithelization Period	Scar Area mm^2	Hydroxy Proline
	4^{th} Day	8^{th} Day	12^{th} Day	16^{th} Day	20^{th} day			
Control	21.67±4.53	38.2±3.56	66.63±3.73	80.23±4.19	83.43±3.24	23.08±1.03	56.84±2.30	1.739±0.03
Povidone Iodine (50mg/animal/day)	39.18±3.72*	58.67±3.853*	88.67±3.69*	90.39±3.89*	98.27±3.58*	18.06±1.48*	37.39±3.21*	4.598±0.04*
Combination	39.47±2.32*	58.12±4.43*	87.38±4.21*	89.64±3.68*	98.61±3.89*	17.89±1.22*	39.60±3.68*	4.684±0.04*

Values are expressed as mean±SE. n =6 in each group. *p≤0.01 is compared to control.

Percent Wound Contraction:

Percent wound contraction refers to the reduction in wound size over time, expressed as a percentage. In the control group, the wound contraction percentages on the 4^{th}, 8^{th}, 12^{th}, 16^{th}, and 20^{th} days were 21.67%, 38.2%, 66.63%, 80.23%, and 83.43% respectively. This indicates that the wound was gradually closing over time in the absence of any treatment.

In the Povidone Iodine group, the wound contraction percentages were higher compared to the control group. On the 4^{th}, 8^{th}, 12^{th}, 16^{th}, and 20^{th} days, the

percentages were 38.18%, 59.67%, 80.67%, 89.39%, and 99.27% respectively. This suggests that Povidone Iodine treatment accelerated wound closure.

Similarly, in the combination treated group, the wound contraction percentages were also higher than the control group. On the 4th, 8th, 12th, 16th, and 20th days, the percentages were 33.47%, 57.12%, 77.38%, 82.64%, and 98.61% respectively.

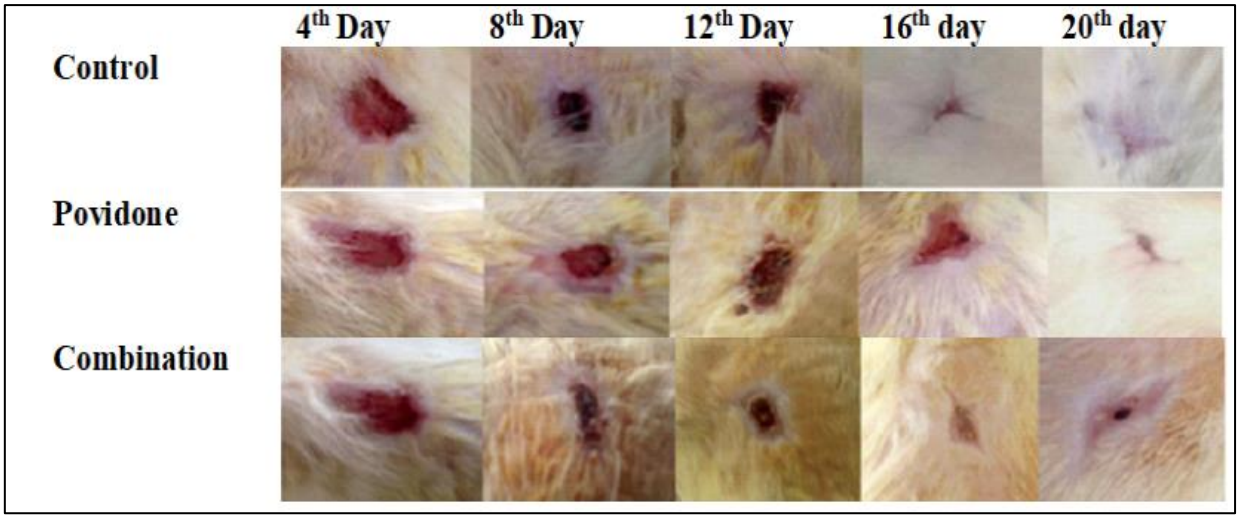

Fig 4: Effect of treatment of Excision wound with Combination on STZ induced diabetic rat

Epithelization Period:

Epithelization refers to the process of new skin cell growth and migration to cover the wound. The epithelization period represents the time taken for complete wound closure. In the control group, the epithelization periods were 23.08 days and 17.89 days for the 8th and 16th day, respectively.

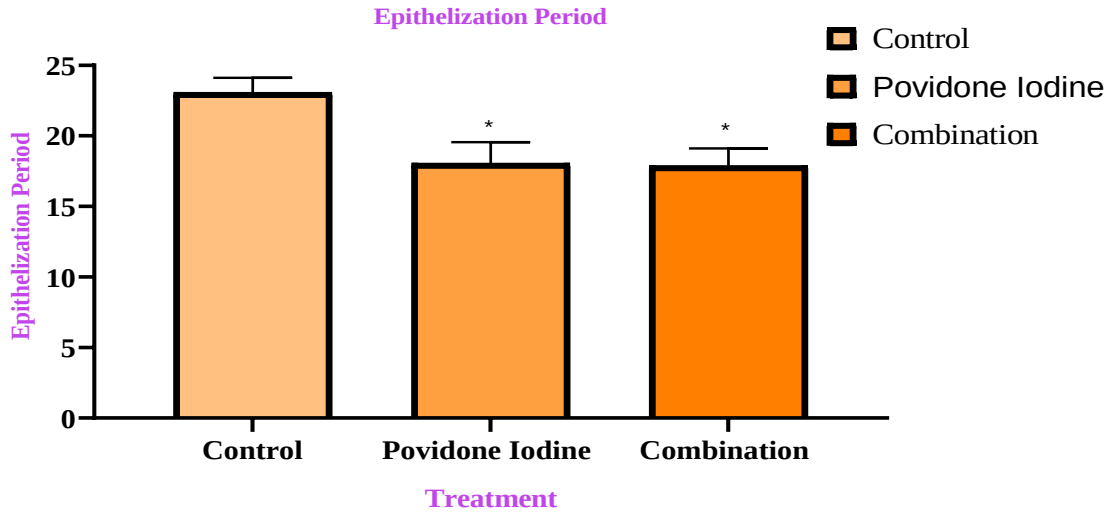

Fig 5: Effect of combination on Epithelization Period

Values are expressed as mean±SE. n =6 in each group. *p≤0.01 is compared to control.

In the Povidone Iodine group, the epithelization periods were shorter (18.06) days, This indicates that Povidone Iodine promoted faster wound healing and epithelization. Similarly, the combination treated group also showed shorter epithelization periods compared to the control group (17.89).

Scar Area:

Scar area represents the extent of scarring left after wound healing. In the control group, the scar areas on the 20th day were measured to be 1.739 mm².

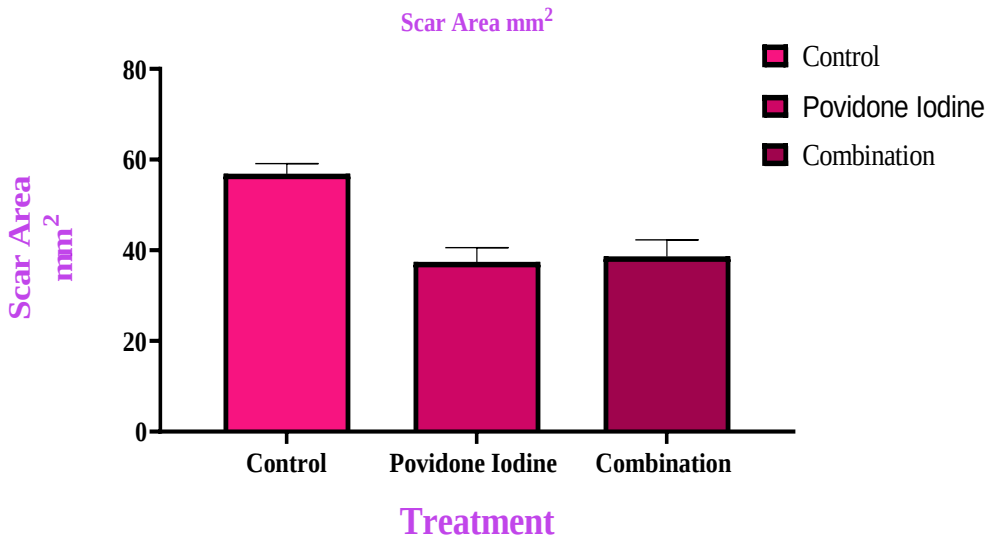

Fig 6: Effect of SBCD on Scar area mm^2
Values are expressed as mean±SE. n =6 in each group. *p≤0.01 is compared to control.

In the Povidone Iodine group, the scar area was larger, measuring 4.598 mm². This suggests that Povidone Iodine treatment may have led to more significant scarring. On the other hand, the combination treated group had a smaller scar area of 3.684 mm² compared to the Povidone Iodine group. The results you provided relate to the effects of different treatments on wound healing parameters in an incision wound model. Let's discuss each parameter and compare the results for the different treatments,

Effect of combination in incision wound model

The topical application of 5% combination ointment increased the breaking strength of the incision wounds to significant extent, i.e. (259.32±4.39) in control was increased up to (432.83±3.44) with Group II (combination) up to (451.37±4.34) with Group III (povidone ointement) (Fig 6). The results were comparable to standard drug povidone iodine.

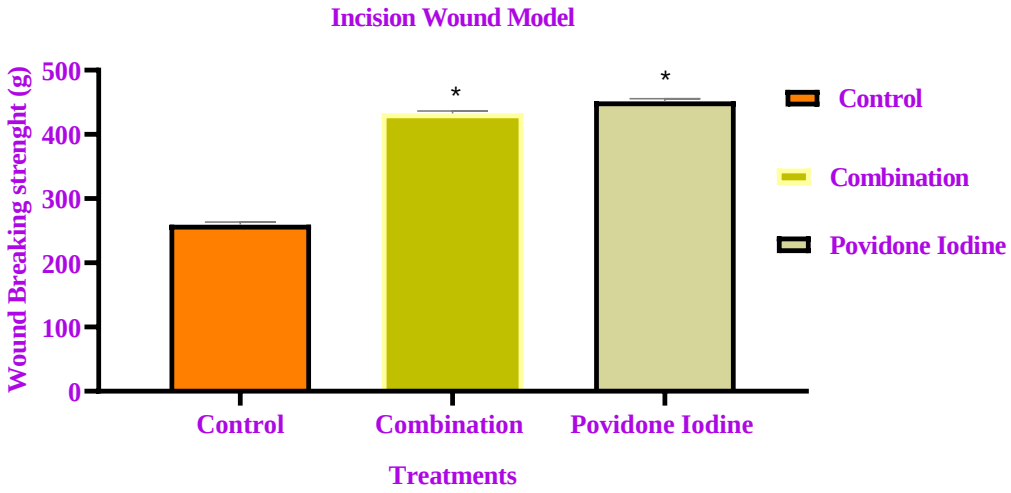

Fig 7: Effect of topical combination treatment on wound breaking strength in gram for Incision wound model
Values are expressed as mean±SE. n =6 in each group. *p≤0.01 is compared to control.

Oral application of combination (25 mg/kg) increased the breaking strength of the incision wounds in Group II to 355.58±3compared to control i.e. 201.32±4.34. It also significantly increased in Group III, i.e. combination (50 mg/kg) to 399.35±3.32.

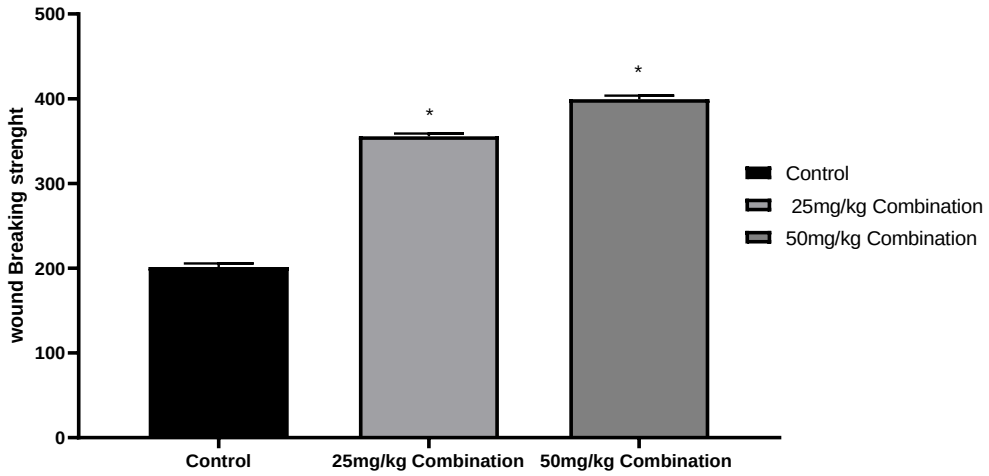

Fig 8: Effect of combination treatment orally on wound breaking strength in gram for Incision wound model

Values are expressed as mean±SE. n =6 in each group. *p≤0.01 is compared to control.

Effect on Dead Space wounds

In the dead space wound study, there was a significant increase in granuloma breaking strength in treated groups at 25 and 50 mg/kg doses, when compared to control.

Treatment	Granuloma breaking strength in grams
Diabetic Control	238.29 ±3.16
Diabetic control + Combination (25mg/kg)	340.83 ± 3.24*
Diabetic control + Combination (50mg/kg)	369.745 ± 3.38*

Values are expressed as mean±SE. n =6 in each group. *p≤0.01 is compared to control.

Hydroxyproline Content (g/ml)

Hydroxyproline content is a marker of collagen synthesis and deposition, indicating the quality and quantity of new tissue formation in the wound. In the control group, the hydroxyproline content was 1.735 g/ml.

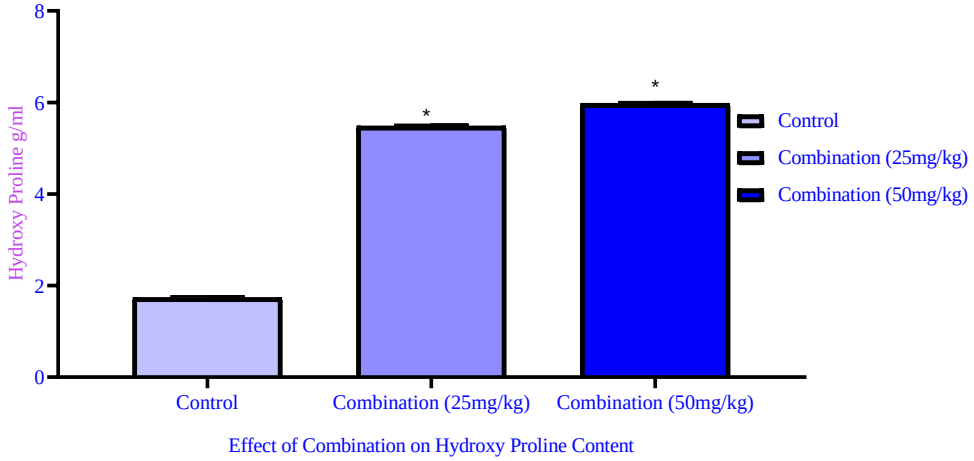

Fig 9: Effect of SBCD on Hydroxyproline content

Values are expressed as mean±SE. n =6 in each group. *p≤0.01 is compared to control.

Treatment with combination at both doses resulted in significantly higher hydroxyproline content compared to the control group. The values were 5.482 ± 0.03* and 5.976± 0.02* 5.482 g/ml for the two doses of combination. These results suggest that combination treatments promote collagen synthesis and deposition, indicating enhanced wound healing.

Hexosamine Content

Hexosamine content is an indicator of total glycosaminoglycan (GAG) levels, which are important components of the extracellular matrix involved in tissue repair. In the control group, the hexosamine content was 0.55g/ml.

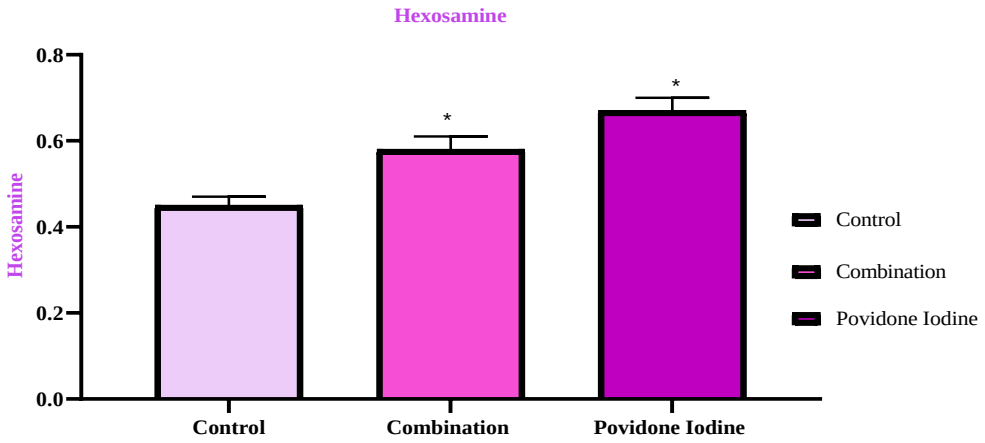

Fig 10: Effect of SBCD on Hexosamine content

Values are expressed as mean±SE. n =6 in each group. *p≤0.01 is compared to control.

Treatment with combination treatments resulted in significantly higher hexosamine content compared to the control group. The values were 0.68± 0.03g/ml and 0.77 g/ml, respectively. These findings suggest that combination treatments enhance the production of glycosaminoglycans, indicating improved tissue repair.

DISCUSSION

The study unveiled a compelling interplay between the phytoconstituents - embelin, berberine, ferulic acid, and asiatic acid, revealing a harmonious synergy that greatly amplified their potential in diabetic wound healing. This dynamic synergy was evident in a spectrum of wound models, surpassing the isolated effects of each constituent. This multifaceted interaction was underpinned by a range of plausible mechanisms that drove the exceptional wound healing outcomes observed.

In the excision wound model, the combination treatment orchestrated a substantial increase in wound contraction, chiefly facilitated by the antioxidants present in these compounds. The collective antioxidant action not only curtailed oxidative stress but also fostered an environment conducive to rapid tissue repair. Moreover, the combination's anti-inflammatory effects were instrumental in reducing inflammation and expediting the healing process. In this regard, the synergy between embelin, berberine, ferulic acid, and asiatic acid harmonized these mechanisms, allowing for enhanced wound closure, quicker tissue regeneration, and minimized scarring [19].

The incision wound model underscored the cooperative strength of the phytoconstituent blend, particularly through its pro-collagen synthesis mechanisms. Ferulic acid, a key component, displayed pronounced effects by promoting collagen synthesis and accelerating tissue remodeling. Simultaneously, the angiogenic properties of asiatic acid stimulated blood vessel formation, ensuring an adequate blood supply for tissue repair. This collaborative effort not only bolstered structural integrity but also expedited the healing process. The synergistic impact between these constituents in harnessing these mechanisms resulted in more robust wound tissues [20].

The dead space wound model further highlighted the synergistic strength of the phytoconstituent combination, primarily through the modulation of inflammation and extracellular matrix components. Embelin's anti-inflammatory properties, working in unison with berberine's antimicrobial effects, fostered an environment conducive to tissue repair. The combined activity of these constituents also influenced the production of glycosaminoglycans (GAGs), crucial for the extracellular matrix. This collaborative effort ensured enhanced tissue strength and efficient tissue healing [21].

Additionally, the biochemical assessments illuminated the profound synergy between these phytoconstituents. The significantly higher hydroxyproline content reflected the cohesive impact of the combination on collagen synthesis and deposition. Ferulic acid, with its collagen-stimulating properties, played a pivotal role. Likewise, the increased hexosamine content, an indicator of GAGs production, was a result of the collective action of these compounds. This synergy was paramount in promoting tissue regeneration and extracellular matrix formation [22].

In summary, the remarkable synergy between embelin, berberine, ferulic acid, and asiatic acid in diabetic wound healing can be attributed to their harmonious interplay of mechanisms. The combination's antioxidant, anti-inflammatory, pro-collagen synthesis, angiogenic, and anti-microbial effects worked in concert to accelerate wound closure, enhance tissue strength, reduce scarring, and promote the production of critical components for tissue repair [23]. This intricate

collaboration of mechanisms underscores the exceptional promise of this phytoconstituent combination as a comprehensive and effective solution for addressing the multifaceted challenges of wound healing in diabetic conditions. Further studies are required for clinical applications and suitable formulation of the combination.

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