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Comparative wound healing potential of *Mitracarpus hirtus* ointment and honey in diabetic albino rats by collagen assessment

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Abstract. *Inalegwu B, Jato JA, Akyengo O, Akighir J. 2021. Comparative wound healing potential of* Mitracarpus hirtus *ointment and honey in diabetic albino rats by collagen assessment. Asian J Nat Prod Biochem 19: 39-44.* All humans will experience some type of wound in every lifetime. Most wounds heal quickly with little or no attention, but many people suffer from complex and/or persistent wounds, therefore posing a burden. This study was designed to assess the efficacy of *Mitracarpus hirtus* (L.) DC. ointment against honey in diabetic rats. To achieve this, percentage wound closure and collagen assessments were used to express treatment efficacy. Results show that on day 21, rats treated with M. hirtus ointment had the highest percentage closure (94.5%) while honey treated and non-treated recorded 90.0% and 83.3% respectively. Similarly, a significant difference ($p < 0.05$) was observed on day 21 in the total collagen deposited in wounds of diabetic rats (10.57 ± 0.7) and *M. hirtus* ointment treated wounds (11.77 ± 0.4) as compared with the non-treated diabetic rats. M. hirtus ointment was efficacious in healing wounds in diabetic rats and heals wound faster than honey and may hold potential for wound healing in diabetes mellitus sufferers. However, the wound healing mechanism of this ointment needs further investigation.

Keyword: Collagen, diabetic rats, honey, *Mitracarpus hirtus,* ointment, wound healing

Abbreviations: DM: Diabetes Mellitus; STZ: Streptozotocin; DMSO: dimethyl sulphuroxide; DC: Diabetic Control; DHT: Diabetic honey treatment; DOT: Diabetic ointment treatment; NSSC: Neutral salt-soluble collagen; PSC: Pepsin soluble collagen; ASC: Acid soluble collagen

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in secretion of insulin, insulin action, or both (ADA 2013). There are three broad categories: type 1, type 2, and gestational diabetes. Although there are other types that are specific to other individual causes (Masharani and German 2011). Symptoms of DM include hyperglycemia, polyuria, polydipsia, and weight loss; sometimes with polyphagia, blurred vision autonomic and peripheral neuropathy which causes poor healing wounds (IDF 2011; Yamany and Sayed 2012).

A wound is defined as a breakdown in the protective function of the skin; the loss of continuity of epithelium, with or without loss of underlying connective tissue following injury to the skin or underlying tissues/organs caused by surgery, a blow, a cut, chemicals, heat/cold, friction shear force, pressure or as a result of disease (Shankar et al. 2014). In the treatment of chronic and other wounds, the primary goals are rapid wound closure and a functional and aesthetically satisfactory scar (Shankar et al. 2014). The wound proceeds through a normal healing cascade of four programmed phases, viz: Homeostasis, inflammation, proliferation and remodelling with specific events to achieve scar formation.

According to Guo and DiPietro (2010), these wound healing events involve: (i) rapid hemostasis; (ii) appropriate inflammation; (iii) mesenchymal cell differentiation, proliferation, and migration to the wound site; (iv) suitable angiogenesis; (v) prompt re-epithelialization (re-growth of epithelial tissue over the wound surface); and (vi) proper synthesis, cross-linking, and alignment of collagen to provide strength to the healing tissue.

Although the phases and events in wound healing are programmed, healing of wounds may delay. Microbial infections, exercise patterns, stress, nutritional status, and diseases such as diabetes have been identified as causes of delay (Damie 2011). Inflammatory and proliferative phases are the most affected stages during delay (Kurahashi and Fujii 2015). These two phases are characterized by neutrophils, macrophages, cytokines, keratinocytes, and fibroblasts, which ultimately result in collagen deposition and transformation of the extracellular matrix (Wong et al. 2013; Olczyk et al. 2014). Assessing the activities of these parameters is informative on progress of wound healing at a molecular level (Zaja-Milatovic and Richmond 2008; Kim et al. 2011; Upadhyay et al. 2014), but assessment of the end product (collagen) is more valuable, in that it is informative on the progress of healing and confirms the expected end products availability (Olczyk et al. 2014). This study used percentage wound closure, collagen deposition, and solubility in various solvents to compare the efficacy of a new herbal formulation (*M. hirtus* ointment) against honey in diabetic rats.

MATERIALS AND METHODS

Preparation of plant extract and ointment

The plant ointment was prepared according to the method detailed in Jato et al. (2018b). Briefly, harvested and identified *Mitracarpus hirtus* (L.) DC. (syn. *Mitracarpus villosus* (Sw.) Cham. & Schltdl. ex DC.) plants were washed and air-dried for 15days. Dried leaves were ground with a Linsan® blending machine (Lin 319) at 1000 RPM for 10 minutes at 25 ^{0}C and methanolic extraction achieved at 250 atm in a rotary evaporator (IKA® EW-28710-22). (50% w/w) *M. hirtus* ointment was prepared by heating 8 g of soft white paraffin in hot water bath for 30 min to melt. Then 8 g of the *M. hirtus* methanolic leave extract was dissolved in 20 mL dimethyl sulphur oxide (DMSO) from JHD® Ltd and mixed thoroughly with the melted paraffin for 20 min. 500 g of the dried leave were ground into fine powder with a Linsan® blending machine (Lin 319) at 1000 RPM for 10 minutes at 25°C.

Source and nature of honey used

500 mL of honey was purchased from a local honey farmer from Kwande Local Government area in Benue State and used for the entire research in its raw undiluted state, although no physicochemical analysis was done on the honey, the honey had a dark brown color and was very thick and sticky especially when allowed to make contact with air for a while.

Experimental animals

Care of animals was in line with National Institute of Health, guide for care and use of laboratory animals (Institute for Laboratory Animal Research 1996). Albino rats purchased from Benue State University Animal House weighing 160-250 g were allowed to acclimatize for 7 days prior to the initiation of the experiment in plastic cages and under laboratory conditions (temperature 22 ± 2 ^{0}C and 12 hr light-dark cycle). Animals were fed with balanced diet purchased from UAC foods Nigeria Ltd and water *ad libitum*.

Experimental design

In this Randomized controlled trial (RCT) study design (3^3), the rate of wound healing based on reduction in wound size and collagen quantification was studied for 21 days with 27 rats on days 7, 14 and 21. The rats were grouped into 3 (n=3) where group 1= control diabetic rats (non treated), group 2= diabetic rats (Honey treated), group =3 diabetic rats ointment treated.

Induction of diabetes mellitus and wound infliction

In fig 1 the flow chart for diabetes induction and wound healing study is presented. According to the method employed by Jato et al. (2018a), 12-hr fasted rats were administered single 65 mg/kg intraperitoneal injection of Streptozotocin (STZ) freshly prepared in 0.1 M sodium citrate buffer (pH 4.5). On day 8 after STZ injection, blood glucose measurement was performed on tail-vein blood with a glucometer (Accu-Chek® Aviva). Rats whose glucose tolerance test and fasting blood glucose levels exceeded 250 mg/dL (13.9 mmol/dL) were considered diabetic, consistent with the findings of (Mendes et al. 2012). The diabetic rats were then inflicted wounds same day to study wound healing by inflicting single full-thickness wounds (2 cm^2) on the dorsum of the rats after disinfection of area with ethanol 90% (Wong et al. 2011).

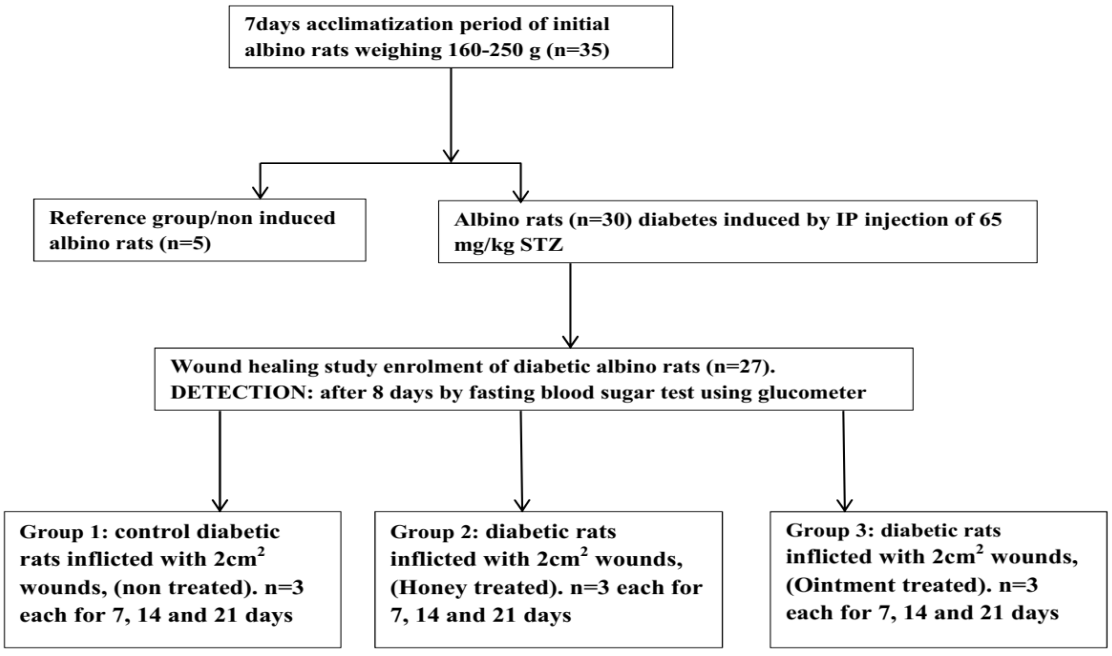

Figure 1. Flow chart for diabetes induction and wound healing study

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Physical examination by measurement of wound size

Wounds were observed postoperatively on days 7, 14 and 21 by measurement of wound size with a rule to determine wound area and then calculating percentage closure as in (eq. 1). Granulated tissues were collected same day for collagen quantification as a measure of wound healing in the diabetic rats as described in Desallais et al. (2014).

$$\%\ wound\ closure = \frac{IWA - CWA}{IWA} \times 100 \quad \text{..... (Eq.1)}$$

Where: IWA: initial wound area; CWA: current wound area

Collagen assay of granulation tissue

The total collagen content in wounds and solubility of collagen in different solvents obtained from granulation tissues in wounds was evaluated by the hydroxyproline assay method of Bergman and Loxly employed by Desallais et al. (2014).

Estimation of total collagen content in granulation tissue

The collagen content in wounds was evaluated by the hydroxyproline assay method of Bergman and Loxly (Desallais et al. 2014). After digestion of punch biopsy specimens (3 mm diameter) in 6 M HCl for 3 hr at 120°C, evaporated to dryness and then made up with a known volume of water. The pH of the samples was adjusted to 7.0. Afterward, samples were mixed with 0.06 M chloramine T and incubated for 20 min at room temperature. Then, 3.15 M perchloric acid and 20% dimethylaminobenzaldehyde added, and samples were incubated for an additional 20 min at 60°C. The absorbance was determined at 557 nm with a spectrophotometer (UV-vis Jenway® 7305).

Extraction of neutral salt-soluble collagen

Granulation tissue was minced, homogenized in 10 vol of neutral salt solvent (1.0 M NaCl, 0.05 M Tris, pH 7.5) containing 20 mM EDTA and 2.0 mM N-ethyl maleimide and stirred for 24 hr. The suspension was then centrifuged at 35,000 g for 1 hr at 4 °C and the extraction was repeated with the pellet. The supernatants were pooled and an assay of hydroxyproline was done.

Extraction of acid-soluble collagen

The residue obtained from neutral salt soluble collagen was re-suspended in 10 vol of 0.5 M acetic acid and extracted for 24 hrs and centrifuged at 5000 g for 10 min. The pellet was then extracted with acetic acid, supernatants were pooled and an aliquot then used for the determination of hydroxyproline as in neutral salt soluble collagen.

Extraction of pepsin-soluble collagen

The residue obtained after acid extraction was re-suspended in 0.5 M acetic acid containing 100 mg pepsin per g of wet tissue. Digestion was carried out for 24 hr followed by centrifugation at 5000 g for 10 min and re-extraction. Aliquots of pooled supernatant were then used for hydroxyproline measurement and aldehyde content in the collagen.

Statistical analysis

SPSS software version 21 was used for analysis of results and is expressed as mean ± S.D. Means were compared statistically using ANOVA and Tukey test as post hoc test. A statistically significant *p*-value <0.05 was considered.

RESULTS AND DISCUSSIONS

Wound healing in treated and non-treated STZ-induced diabetic albino rats as indicated by % wound closure

In Figure 2, wound healing results in albino rats treated as measured by percentage wound closure are presented. On day 7, the diabetic rats treated with *M. hirtus* ointment recorded the highest percentage closure of 14.50% while rats treated with honey recorded the least closure (3.3%). On day 14, the non-treated diabetic group (16.3%) whereby they showed lesser percentage of wound closure than the honey-treated rats (32.5%) while the *M. hirtus* ointment treated group maintained the lead with a percentage closure of 45.0%. On day 21, the diabetic rats treated with *M. hirtus* ointment maintained highest percentage closure of 94.5%. The change observed on day 14 was also maintained, an 83.3% and 90.0% wound closure was recorded for non-treated diabetic rats and diabetic rats treated with honey respectively. In all, wound closure and scar formation were recorded on days 16, 17 and 19 for diabetic ointment treatment, diabetic honey treatment, and diabetic control.

Wound healing in treated and non-treated STZ-induced diabetic albino rats as indicated by assessment of collagen formation

Table 1 presents results of total collagen deposited in wounds of treated and non-treated diabetic rats. They show that there is no statistically significant ($p < 0.05$) increase in the total collagen deposited in wounds of diabetic rats treated with *M. hirtus* ointment and with honey at day 7 post wound excision. A statistically significant difference ($p < 0.05$) was observed at day 14 in the deposition of total collagen for diabetic rats treated with honey (5.53 ± 0.2) and diabetic rats treated with *M. hirtus* ointment (5.67 ± 0.3) as compared with the non-treated diabetic rats (5.00 ± 0.1). Similarly, a significant ($p < 0.05$) increase was observed at day 21 in the deposition of total collagen in honey-treated wounds of diabetic rats (10.57 ± 0.7) and *M. hirtus* ointment treated wounds (11.77 ± 0.4) as compared with the non-treated diabetic rats. Thus all diabetic rats had increased total collagen formation with increased days of healing.

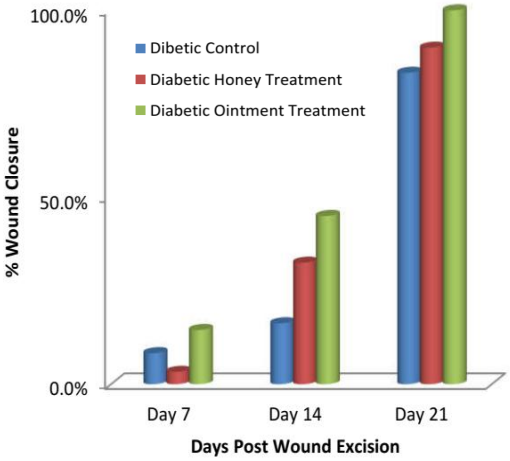

Figure 2. Wound healing in albino rats treated as measured by percentage wound closure

Table 1. Total collagen deposited at 7, 14, and 21 days post wound excision in STZ-induced diabetic rats in various treatment groups

Group	Duration of wounds		
	Day 7	Day 14	Day 21
DC	1.70 ± 0.4[a]	5.00 ± 0.1[a*]	9.83 ± 0.4 [a*]
DHT	1.93 ± 0.9 [a]	5.53 ± 0.2[b]	10.57 ± 0.7[ab]
DOT	2.40 ± 0.4[a]	5.67 ± 0.3[b]	11.77 ± 0.4[b]

DC-Diabetic Control, DHT-Diabetic honey treatment, and DOT-Diabetic ointment treatment. Data are expressed as the mean ± SD from three animals in each group. Significant differences between the diabetic control and treated groups were assessed using Turkey test. *: significant difference ($p < 0.05$), [a, b]: values with same alphabets belongs to the same Homogeneous subset

Tables 2, 3 and 4 show solubility of collagen in various solvents namely: acid, neutral salt and pepsin respectively for the treated and non-treated groups. Results show that, no significant ($p < 0.05$) decrease was recorded in the pepsin soluble collagen (PSC) and neutral salt soluble collagen (NSSC) at day 7, 14 and 21 in the diabetic rats treated with honey, and diabetic rats treated with *M. hirtus* ointment when compared to the non-treated diabetic rats. Conversely, a significant ($p < 0.05$) decrease was observed in the solubility of acid-soluble collagen (ASC) on days 7, 14 and 21 in the diabetic rats treated with honey, and diabetic rats treated with *M. hirtus* ointment when compared to the non-treated diabetic rats. There was a decreasing trend in the solubility of ASC, NSSC and PSC in all the groups, indicating maturation and toughness of collagen deposits through the healing period. Therefore, in all the groups of diabetic rats there was a decrease in solubility with increase in days of experiment.

The *M. hirtus* ointment-treated diabetic rat wounds showed higher percentage closure than the honey-treated diabetic wounds in the albino rats while the non-treated diabetic rat wounds recorded the least percentage closure. The findings agree with that of Dwivedi and Chaudhary (2012) on ampucure in diabetic wounds in rats while the findings of Barua et al. (2013) (89.76%) agree with findings using honey on day 21. Also, Sazegar et al. (2011) and Nisbet et al. (2010) reported a significant percentage closure in honey-treated wounds than the non-treated wounds.

Table 2. Wound healing in treated and non-treated STZ-induced diabetic albino rats as indicated by solubility of collagen in acid

Days	7	14	21
Group 1: DC (Mean ± SD)	524.00 ± 11.10[b]	506.00 ± 9.00[b]	492.67 ± 11.40[b]
Group 2: DHT (Mean ± SD)	513.67 ± 5.70[b*]	499.00 ± 7.00[b]	473.67 ± 13.10[b*]
Group 3: DOT (Mean ± SD)	393.67 ± 11.00[a*]	356.67 ± 49.00[a*]	342.00 ± 44.00[a*]

Note: DC-Diabetic Control, DHT-Diabetic honey treatment, DOT-Diabetic ointment treatment. *: significantly different down the column at $p < 0.05$ when compared to the control, Values with the same alphabetical superscripts ([a, b]) belong to the same Homogeneous subset as compared by Turkey post Hoc test

Table 3. Wound healing in treated and non-treated STZ-induced diabetic albino rats as indicated by solubility of collagen in neutral salt

Days	7	14	21
Group 1: DC (Mean ± SD)	116.67 ± 16.80[a]	92.33 ± 10.80[a]	78.67 ± 8.10[a]
Group 2: DHT (Mean ± SD)	106.0 ± 4.00[a]	85.33 ± 13.60[a]	73.00 ± 9.60[a]
Group 3: DOT (Mean ± SD)	101.33 ± 6.80[a]	80.67 ± 13.60[a]	68.33 ± 9.00[a]

Note: DC-Diabetic Control, DHT-Diabetic honey treatment, DOT-Diabetic ointment treatment. *: significantly different down the column at $p < 0.05$ when compared to the control, Values with the same alphabetical superscripts ([a, b]) belong to the same Homogeneous subset as compared by Turkey post Hoc test

Table 4. Wound healing in treated and non-treated STZ-induced diabetic albino rats as indicated by solubility of collagen in pepsin

Days	7	14	21
Group 1: DC (Mean ± SD)	2730.33 ± 333.00[a]	2480.00 ± 385.10[a]	2349.00 ± 201.80[a]
Group 2: DHT (Mean ± SD)	2483.00 ± 404.50[a]	2266.33 ± 185.00[a]	2188.33 ± 424.90[a]
Group 3: DOT (Mean ± SD)	2278.67 ± 294.40[a]	2075.33 ± 168.20[a]	1996.00 ± 314.00[a]

Note: DC-Diabetic Control, DHT-Diabetic honey treatment, DOT-Diabetic ointment treatment. *: significantly different down the column at $p < 0.05$ when compared to the control, Values with the same alphabetical superscripts ([a, b]) belong to the same Homogeneous subset as compared by Turkey post Hoc test

The higher percentage closure observed in the treated wounds of the diabetic rats can be attributed to increased deposition of total collagen and reduction in oxidative stress arising from the infliction of wounds to the rats. Close interaction of cells is necessary for synthesis and deposition of collagen facilitated by contracture of the skin tissue (Lembong et al. 2017). Cardiac glycosides have been implicated for contraction in tissues by inhibiting the Na^+-K^+ ATPase pump in the sarcolemmal membrane of the myocyte and other cells (Cunningham et al. 2018). This inhibition causes intracellular accumulation of Na^+, which makes the Na^+-Ca^{2+} pump extrude less Ca^{2+}, causing Ca^{2+} to accumulate inside the cell resulting in increased force of contraction necessary for wound closure (Lembong et al. 2017). In addition to excellent water-absorbing quality, biodegradability, biocompatibility and outstanding film-forming properties, interactions of Ca^{2+} ions with the cells to facilitate contractions are the basis for using calcium alginate in wound dressing (Wang et al. 2015). Therefore, we submit that increased closures associated with the plant ointment may be the effect of cardiac glycosides in the plant on the wounds.

Collagen obtained from the treated and non-treated diabetic rats showed decreased solubility in the various solvents through the days of treatment. Also, the collagen of *M. hirtus* and honey exhibited lesser solubility in the acid, neutral salt buffer and pepsin solvents. This decrease in solubility was a result of collagen maturation (Weston and Althen 2002). The mature collagens have lesser crosslinks because the initial collagen produced is reducible and over time are replaced by mature, thermally stable and less soluble crosslinks. These mature crosslinks rather than total collagen are the key factors in collagen-related toughness (Weston and Althen 2002; Ponrasu and Suguna 2014). In an earlier submission, we reported that there was decreased antioxidant enzyme activity associated with increased wound healing. The development of mature tough collagen and the epidermal layer stratum corneum have been implicated for this negative correlation and the resultant challenges in transdermal drug delivery (Yang et al. 2018; Jato et al. 2021). Results obtained for the acid-soluble collagen were in agreement with the findings of Ponrasu and Suguna (2014) and Ponrasu et al. (2018) while that of the NSSC was in agreement with reports by Kirubanandan et al. (2013) and Ponrasu and Suguna (2014). PSC also recorded very high solubility more than the ASC and NSSC as observed by other studies (Ponrasu and Suguna 2014; Ponrasu et al. 2018; Kirubanandan et al. 2013). This is because the acid and the neutral salt only carry out partial solubility, while the insoluble collagen is soluble in pepsin (Kirubanandan et al. 2013).

In conclusion, from the findings in this study, it was concluded that wounds of diabetic rats treated with *M. hirtus* ointment healed faster than honey. Thus diabetic rats treated with the ointment recorded the highest percentage of wound closure. Also, total collagen deposited in diabetic rats was highest in the ointment treated rats followed by honey treated rats while the non-treated was least and the solubility of collagen in the various solvents was decreased concomitantly with increase in healing and was lesser in the ointment treated diabetic rats, suggesting faster maturation of the deposited collagen. It is our view however that, the mechanism of action of the ointment be studied in addition to the identification and isolation of the bioactive compounds responsible for the efficacy of the ointment.

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