Chronic Diseases Journal

DOI: 10.22122/cdj.v9i1.581 Published by Vesnu Publications

Antioxidant enzymes in assessment of wound healing efficacy of Mitracarpus villosus ointment in diabetic state

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Original Article

Abstract

BACKGROUND: Diabetes mellitus (DM) is a global health problem that accounted for about 1.5 million deaths in 2012; majority of these deaths are associated with complications such as poor wound healing. Assessment and management of wounds in people with DM has been identified as the major limiting factor besides poorly-managed hyperglycaemia.

METHODS: To determine the effect of Mitracarpus villosus (M. villosus) ointment in healing of wounds in diabetic albino rats as compared to honey, the antioxidant enzymes of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were used as assessment parameters in three groups (n = 3) for a period of 21 days.

RESULTS: A significant decrease ($P < 0.05$) was observed in the enzymes' activity at days 7, 14, and 21 for both SOD and GPx in the treated groups, especially for M. villosus ointment treatment as compared to the non-treated group. In addition, there was no significance to CAT decrease 7 days after wound excision in the treatment groups as compared to the non-treated diabetic rats (11.66 ± 0.90, 12.20 ± 0.40, 13.30 ± 2.19) for control, honey, and ointment treatments, respectively. Physical assessment showed that reduced wound size was recorded more in the ointment-treated group than honey-treated and non-treated groups.

CONCLUSION: M. villosus ointment can heal wounds faster than honey and holds potential for wound healing in DM sufferers, and exacerbated tissue stress can be ameliorated using M. villosus ointment. However, the isolation and characterisation of specific bioactive compounds in the ointment responsible for specific enzyme activities, the effect of ointment on collagen synthesis, and mechanism by which wound healing is achieved requires further studies.

KEYWORDS: Diabetes Mellitus; Superoxide Dismutase; Glutathione Peroxidase; Catalase; Diabetic Complications

Date of submission: 13 Mar. 2020, *Date of acceptance:* 20 Sep. 2020

Citation: Jato JA ،Inalegwu B ،Onyezili FN. **Antioxidant enzymes in assessment of wound healing efficacy of Mitracarpus villosus ointment in diabetic state.** Chron Dis J 2021; 9(1): 7-13.

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterised by hyperglycaemia resulting from poor or non-secretion of insulin, insulin action, or both.[1] Three major categories are notable: gestational, type 1, and type 2 which is the most prevalent, accounting for about 90% of all cases.[2]

In 2012, DM accounted for about 1.5 million deaths all over the world.[3] Majority of DM deaths are due to secondary causes other than hyperglycaemia, often referred to as complications.[4] These complications arising from poorly-managed DM include but not all: atherosclerosis-related disease such as coronary artery disease (CAD), peripheral vascular disease (PVD), and stroke classified as macrovascular diseases. The second class of microvascular damages include retinopathy, nephropathy, and neuropathy, which are the leading causes of blindness, renal failure, and nerve injuries related to non-traumatic amputations and non-healing ulcers.[5,6]

Tremendous efforts have been made in early diagnosis and management of DM and

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the search for a permanent cure.[3] However, much still needs to be done in the area of assessing and managing complications such as non-healing wounds/ulcers that occur in patients with poorly-managed DM.[7]

The knowledge of Mitracarpus villosus (M. villosus) - a Rubiaceae family member used in traditional wound healing - led to its use in this study. It is a common annual erect weed that grows up to 60 cm tall especially in tropical Asia and Africa.[8,9] The stem is angled, hairy, and sparsely branched. It is woody at the base, segmented into nodes with each internode bearing a pair of leaves.[8] The fresh leaves are green in colour with a mild odour, peppery, and with the characteristic bitter taste of saponins. Studies have shown that aqueous, ethanolic, methanolic, and a combination of solvent extracts of M. villosus contain alkaloids, saponins, tannins, flavonoids, phenolics, glycosides, resins, steroids, and terpenoids.[8,10,11] Further look into these studies shows consistent presences of saponins, tannins, flavonoids, and phenolics in M. villosus irrespective of the solvent extraction and plant part used. The consistency of these compounds, antioxidant, antibactericidal, antipyretic wound healing activities and their utilization in folk medicine informed our choice of *M.* villosus in this study.[12-15]

Antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxide (GPx), and catalase (CAT) prove promising DM wounds assessment since they have been utilized in assessment of wound healing.[16] This study was designed to determine the wound healing potential of M. villosus ointment in diabetic albino rats as compared to honey using SOD, GPx, and CAT as assessment parameters in addition to physical examination.[17-19]

Materials and Methods

Preparation of plant extract and ointment: The plant extract was prepared according to the method detailed in Jato et al.[20] Briefly, 50% w/w *M.* villosus ointment was prepared by heating 8 g of white soft paraffin in hot water bath for 30 minutes to melt. 8 g of M. villosus methanolic leaves extract was dissolved in 20 ml dimethyl sulfoxide (DMSO) from JHD® Ltd. (China) and mixed thoroughly with the melted paraffin for 20 minutes. The obtained ointment was stored in tubes and then desiccator for further use.

Animal care and groupings: Care of animals was in line with National Institutes of Health guide for care and use of laboratory animals.[21] Albino rats weighing 160-250 g were obtained from animal house of Benue State University, Makurdi, Benue State, Nigeria. A 7-day acclimatization period prior to initiation of experiment under laboratory conditions was permitted. Animals were fed with balanced diet from UAC foods limited Nigeria and water ad libitum. In this randomized controlled trial (RCT) study design,[3] experimental animals had blood sampled through cardiac puncture into heparin tubes on days 7, 14, and 21 post-injury from three groups (n = 3) as presented below:

Group 1 = Diabetic control (DC)
Group 2 = Diabetic honey treatment (DHT)
Group 3 = Diabetic ointment treatment (DOT)

Diabetes induction, infliction, and measurement of wounds in diabetic albino rats: According to the method employed by Jato et al.,[22] 30 rats fasted for 12 hours were administered single 65 mg/kg intraperitoneal (IP) injection of streptozotocin (STZ) freshly prepared in 0.1M sodium citrate buffer (pH = 4.5). 8 days after diabetes induction, blood glucose was measured with a glucometer (Accu-Chek® Aviva). Rats whose glucose tolerance test and fating blood glucose levels exceeded 250 mg/dl (13.9 mmol/dl) were considered diabetic, consistent with other findings.[23] Diabetic rats (n = 27) were then inflicted 2 cm^2 single full-thickness wounds on their dorsum same day to study wound healing, whereby wound sizes were measured using a meter

ruler and the percentage of healing was calculated (Equation 1).[24]

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

IWA = Initial wound area; CWA = Current wound area

SOD and GPx assay: The activities of SOD and GPx were measured by colorimetric method with a commercial kit (RANSOD, Randox Co., UK). According to manufacturer's manual, 0.5 ml whole blood was centrifuged for 10 minutes at 700 g and the plasma was aspirated off. The erythrocytes were washed four times with 3 ml of 0.9% sodium chloride (NaCl) solution and were centrifuged each time to separate the supernatant. The washed erythrocytes were diluted to 2 ml with cold redistilled water. After thorough mixing, the lysate was diluted with 0.01 mol/l phosphate buffer (pH = 7) with a final dilution factor of 200. The reagents were then added to the diluted samples.

Xanthine and xanthine oxidase (XO) were employed to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity in the hemolysate was determined by the degree of inhibition of this reaction, as one unit of SOD corresponds to 50% inhibition of INT reduction under assay condition. Finally, the enzyme activity was measured at 505 nm and expressed as units/mg of haemoglobin (Hb).

GPx activity was measured by a commercial kit from the same source. According to the manual, 0.05 ml of whole blood was diluted and incubated with 3 ml of diluting agent to form the hemolysate. The GPx present in the hemolysate catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of GSH reductase (GR) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), the oxidized GSH is immediately converted to reduced form with a concomitant oxidation of NADPH to $NADP^+$. The absorbance was measured at 340 nm and results were expressed as units/mg of Hb.

CAT assay: The CAT activity was measured spectrophotometrically by monitoring the decomposition of hydrogen peroxide (H_2O_2) using the procedure of Aebi.[25] Briefly, 0.5 ml of 30 mmol/l H_2O_2 solution in 50 mmol/l phosphate buffer (pH = 7.0) and 1 ml of 1:10 diluted erythrocyte lysates were added and the consumption of H_2O_2 was followed spectrophotometrically at 240 nm for 2 minutes at 25 °C. The molar extinction coefficient was 43.6 l/mol per centimeter for H_2O_2. CAT activity was expressed as the unit then was defined as µmol H_2O_2 consumed/minute per milligram Hb.

Statistical analysis: Results were analysed statistically and means were compared by analysis of variance (ANOVA) and Tukey's post hoc test using SPSS software (version 21, IBM Corporation, Armonk, NY, USA). Results were then expressed as mean ± standard deviation (SD) and a P-value of < 0.05 was considered statistically significant.

Research and ethics committee approval: This research was approved by the Research and Ethics Committee of the Federal University of Agriculture Makurdi, Makurdi, Nigeria, with the approval letter ref: FUAM/REC/19/1253612.

Results

Figure 1 presents images of wound healing in diabetic albino rats, treated with M. villosus ointment. Images show that the wounds of diabetic rats treated with M. villosus ointment healed faster than non-treated and the honey-treated diabetic rats. Wounds on non-treated rats healed the most slowly.

The activity of SOD in STZ-induced diabetic rats with treated and non-treated wounds are presented in table 1. Results showed that there was a significant ($P < 0.05$) decrease in the activity of the enzyme in diabetic rats treated with honey and the diabetic rats treated with *M.* villosus ointment (12.60 ± 0.28) when

compared with the non-treated diabetic rats (11.02 ± 0.85) on day 7. There was a statistically significant ($P < 0.05$) decrease in the enzyme activity in the two treatment groups when compared to the non-treated group on day 14.

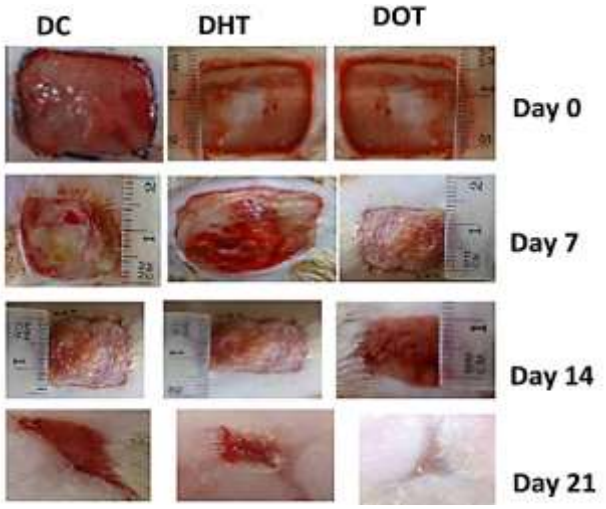

Figure 1. Images of wound healing in diabetic albino rats treated with Mitracarpus villosus (M. villosus) ointment and with honey

DC: Diabetic control; DHT: Diabetic honey treatment; DOT: Diabetic ointment treatment

A significant ($P < 0.05$) decrease in the enzyme activity was observed only in the diabetic rats (3.42 ± 0.49) on day 21. A decreased activity was observed in all the groups with increase in closure. Therefore, M. villosus ointment had greater effect than honey on the activity of SOD in diabetic rats inflicted with wounds.

Table 1. Superoxide dismutase (SOD) activity at 7, 14, and 21 days after wound excision in treated and non-treated streptozotocin (STZ)-induced diabetic rats as a measure of wound healing

Days	Group 1	Group 2	Group 3
	Mean ± SD	Mean ± SD	Mean ± SD
7	11.02 ± 0.85^{a}	$11.67 \pm 0.61^{ab*}$	$12.60 \pm 0.28^{b*}$
14	6.55 ± 0.97^{a}	7.62 ± 0.70^{a}	8.48 ± 1.15^{a}
21	3.42 ± 0.49^{a}	$4.57 \pm 0.80^{ab*}$	$5.45 \pm 0.63^{a*}$

*Significantly different across the row 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 Tukey's post hoc test;SD: Standard deviation

In table 2, the activity of CAT in STZ-induced diabetic rats with treated and non-treated wounds is presented. There was no significant ($P < 0.05$) decrease in the activity of CAT in the treated diabetic rats when compared to the non-treated rats on day 7 after wound excision. On day 14 after wound excision, a significant ($P < 0.05$) decrease was recorded in the activity of CAT in diabetic rats treated with *M.* villosus ointment (11.51 ± 1.33) when compared to the non-treated diabetic rats (8.32 ± 0.70) as was observed in the diabetic rats treated with honey (9.45 ± 0.71). On day 21 after wound excision, no significant ($P < 0.05$) decrease was recorded in the diabetic rats treated with honey (5.23 ± 1.16) as compared to non-treated diabetic rats (3.44 ± 0.57), while a significant difference was observed in the rats treated with M. villosus ointment (7.65 ± 1.17). Therefore, for all groups of the diabetic rats inflicted with wounds, a reduction in the activity of the CAT was observed with increase in number of days after wound excision.

Table 2. Catalase (CAT) activity at 7, 14, and 21 days after wound excision in treated and non-treated streptozotocin (STZ)-induced diabetic rats as a measure of wound healing

Days	Group 1	Group 2	Group 3
	Mean ± SD	Mean ± SD	Mean ± SD
7	11.66 ± 0.90^{a}	12.20 ± 0.40^{a}	13.30 ± 2.19^{a}
14	8.32 ± 0.70^{a}	$9.45 \pm 0.71^{ab*}$	$11.51 \pm 1.33^{b*}$
21	3.44 ± 0.57^{a}	5.23 ± 1.16^{ab}	$7.65 \pm 1.17^{b*}$

* Significantly different across the row 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 Tukey's post hoc test
SD: Standard deviation

In table 3, the activity of GPx in STZ-induced diabetic rats with treated and non-treated wounds is presented. It shows that there is a significant ($P < 0.05$) decrease in the GPx activity in diabetic rats treated with honey on day 7 (3.34 ± 0.31), day 14 (2.67 ± 0.16), and day 21 (1.61 ± 0.13) when compared with the

non-treated diabetic rats on day 7 (2.28 ± 0.36), day 14 (1.67 ± 0.13), and day 21 (1.12 ± 0.25), respectively. Similarly, there was a significant ($P < 0.05$) decrease in the diabetic rats treated with *M.* villosus ointment on day 7 (4.17 ± 0.20), day 14 (3.34 ± 0.31), and day 21 (2.75 ± 0.30) as compared to the diabetic rats with non-treated wounds. Therefore, for all groups of the diabetic rats inflicted with wounds, a reduction in the activity of the GPx was observed with increase in number of days after wound excision.

Table 3. Glutathione peroxidase (GPx) activity at 7, 14, and 21 days after wound excision in treated and non-treated streptozotocin (STZ)-induced diabetic rats as a measure of wound healing

Days	Group 1	Group 2	Group 3
	Mean ± SD	Mean ± SD	Mean ± SD
7	2.28 ± 0.36[a]	3.34 ± 0.31[b*]	4.17 ± 0.20[c*]
14	1.67 ± 0.13[a]	2.67 ± 0.16[b*]	3.34 ± 0.31[c*]
21	1.12 ± 0.25[a]	1.61 ± 0.13[a*]	2.75 ± 0.30[b*]

* Significantly different across the row 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 Tukey's post hoc test
SD: Standard deviation

Furthermore, the activities of SOD, CAT, and GPx were raised at the early stage of wounds but reduced significantly with healing in wounds. This suggests a negative correlation between the presence of wound and enzyme activity. In addition, results showed a negative correlation in SOD and GPx activity per each group through the experimental period.

Discussion

The activity of SOD was raised in the treated and non-treated diabetic rats and increased with an increase in the healing and increase in number of days after wound excision. This is the result of reduced oxidative stress which accompanied increase in healing. The findings in the activity of SOD for the rats treated with M. villosus ointment are in agreement with the reports of Bhor et al.[26] for treated groups (12.95 ± 1.37). This also agrees with other findings for stressed animals.[27,28] Possible reasons for this decrease in enzyme activity is decreased reactive oxygen species (ROS) in animals.

CAT activity also showed raised levels at day 7 in the treated and non-treated diabetic rats and decreased with increase in healing and increase in number of days after wound excision. These findings agree with the findings of similar studies (21.6 µmol/min^{-1}/gHb^{-1}) for hyperglycaemic group,[29] (14.73 ± 2.88 U/mg) for diabetic rats,[30] and (17.5 U/ml) for non-treated wound of type 2 diabetic rats.[31] CAT is involved in the elimination of H_2O_2. As an antioxidant enzyme, CAT is interconnected in function to the common course of decreasing the accumulation of lipid peroxides and decreasing oxidative stress in wounded rats.[32]

The activity of GPx also showed raised levels at day 7 in the treated and non-treated diabetic rats and decreased levels with an increase in healing and increase in the number of days after wound excision. Our findings confirmed other studies (1.54 ± 0.05 and 1.67 ± 0.07) for the diabetic control and diabetic treated groups, respectively,[27] (6.4 µmol/min^{-1}/gHb^{-1}) in a hyperglycaemic group.[29] Possible reason for the increase in activity of these antioxidant enzymes observed in the treated rats may be that the bioactive compounds in the wound sites triggered the release of these enzymes for faster healing of wounds.[29] Increased healing is often associated with therapies that reduce lipid peroxidation and other oxidative stress. Honey is reported to be effective in this aspect,[33] due in part, to the report by Jato et al.[20] and several other authors of the presence of similar phytochemicals with informed antioxidant activities in M. villosus, such as found in honey. Proposition is, therefore, made that M. villosus ointment and honey have similar wound healing mechanism.

One such class of phytochemicals is flavonoids and has been implicated for wound healing in diabetic rats.[28] Agbafor and

Emmanuel[28] are of the view that flavonoids elicit this healing by: (1) shortening the inflammatory phase and (2) reducing the oxidative stress associated with healing. This effect will lead ultimately in the synthesis and deposition of collagen, remodelling of healed wound. Studies have shown that transdermal drug delivery across the skin faces challenges due to the epidermal layer stratum corneum.[34] Authors are of the view that the general decrease in the activity of antioxidant enzymes (SOD, CAT, and GPx) as observed from day 7 to day 21 can be attributed to decreased ointment and honey movement across the skin due to gradual re-establishment of skin anatomy and physiology resulting in wound healing.

This research faced limitation in addressing which compound in M. villosus ointment precisely was responsible for wound healing and enzyme activity. Thus, it is recommended that further studies on isolation and identification of specific compound action in M. villosus ointment using techniques such as high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS), be embarked upon. Also, carcinogenic possibility over chronic use needs to be determined.

Conclusion

From the findings in this study, it is concluded that *M.* villosus ointment heals wounds in diabetic rats, and that it heals wounds of diabetic rats faster than honey based on physical assessment. M. villosus also reduces the high levels of the antioxidant enzymes of SOD, CAT, and GPx which are often raised in stress conditions such as wounds, inferring that healing took place by enzymatic assessment. Therefore, recommendations are made that the plant ointment be further studied for its ability to cause collagen synthesis and other mechanisms by which it elicits wound healing.

Conflict of Interests

Authors have no conflict of interests.

Acknowledgments

Authors are thankful to Mr. Itolo Ogli and Mr. Vincent Upev, both of University of Agriculture Makurdi, for assistance with some reagents.

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