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Abstract

Background: This study was designed to ascertain this claim and to investigate the possible mechanism of action in relation to their phytochemical contents, using crude extracts of *N. latifolia* and *M. esculenta* leaves on topical wound in a rat model of Type 1 diabetes.

Methods and Materials: The leaves were air-dried under shade and subjected to cold extraction in 95% ethanol. Normoglycaemic male Wistar rats were subjected to 2-month high-energy diet/ fat emulsion manipulation and injected with alloxan (150 mg/kg BW/day, *i. p.*). Fasting plasma glucose was determined after 7 days and rats with values exceeding 200 mg/dl were selected. 16 diabetic rats (120 – 180 g) were randomly assigned to four groups of four animals each. Wound was inflicted on the back of each rat by excision method. The crude extracts were topically applied over a period of 21 days. The scar tissues were removed at 7 days interval for collagen quantification and wound margin reduction was also monitored using tracing paper.

Results: The crude extracts showed efficient wound healing activity as revealed by increased collagen content (scar tissues *versus* fresh wound tissues *ab initio*). Percentage wound closure also progressed significantly ($p < 0.05$) upon topical application of the extracts.

Conclusion: The crude extract of *N. latifolia* leaves has proven to be more potent than that of *M. esculenta* in wound healing in Type I diabetic rats. It stimulated wound contraction and collagen formation, making it a promising natural product for further screening in search of new chemical entities that can be useful in diabetic wound management.

Key words: Type 1 diabetes, collagen content, wound healing, anthocyanins, phenolic compounds

Introduction

It is estimated that > 100, 000 people die annually in India, and between 40, 000 – 99, 999 people die in Nigeria from diabetes-related complications (International Diabetes Federation's – IDF Atlas, 2011). The 6th edition of the Diabetes Atlas's new figures indicated that the number of people living with diabetes in the world is expected to rise from 366 million in 2011 to 552 million by 2030. India continues to be the "Diabetes Capital" of the world with Gujarat state having high prevalence of the dreaded disease, while over 5 million people suffer from the disease in Africa and the number is expected to increase to 15 million by 2025 (Joshi and Parikh, 2007, Kaveeshwar and Cornwall 2014).

According to the American Diabetes Association, 25% of people with diabetes will suffer from a wound problem during their lifetime, and it has been estimated that lower limb amputations in diabetic patients account for healing associated with multitude of factors, including neuropathy, vascular disease, and foot deformities (Jude *et al.*, 1998). At the cellular level, an increase in the number of acute inflammatory cells, absence of cellular growth, and migration of the epidermis have been observed (Ferguson *et al.*, 1996). Patients with diabetes have impaired leukocyte function, and the metabolic abnormalities of diabetes lead to inadequate migration of neutrophils and macrophages to the wound, along with reduced chemotaxis (Delamaire *et al.*, 1997). Such cellular changes would predispose individuals to an increased risk of wound infection.

Medicinal plants used to treat hypoglycemic or hyperglycemic conditions are of considerable interest for ethno-botanical community as they are recognized to contain valuable medicinal properties in different parts of the plant and a number of plants have shown varying degree of hypoglycemic and anti-hyperglycemic activity (Grover *et al.*, 2002). The active principles of many plant species are isolated for direct use as drugs, lead compounds or pharmacological agents (Fabricant and Farnsworth, 2001). Several species of medicinal plants are used in the treatment of diabetes mellitus, a disease affecting large number of people world-wide. Traditional plant medicines or herbal formulations might offer a natural key to managing diabetic complications. Wound healing, or cicatrization, is an intricate process in which the skin (or another organ-tissue) repairs itself after injury (Murphy *et al.*, 2009). When tissue is first wounded, blood comes in contact with collagen, triggering blood platelets to begin secreting inflammatory factors. Platelets also express glycoproteins on their cell membranes that allow them to stick to one another and to aggregate, forming a mass (Midwood *et al.*, 2004).

Materials and Methods

Plant Materials

Manihot esculenta leaves were collected from a local farm in Oke-Baale, Osogbo, Osun State, Nigeria while *Nauclea latifolia* leaves were collected from Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria. They were identified and deposited at the herbarium of the Department of Plant Biological, University of Ilorin, Ilorin, Kwara State, Nigeria – UIH002/1094 and UIH004/506, respectively.

Animals

Wistar rats were purchased at the Animal House, Department of Anatomy, University of Ibadan, Ibadan, Oyo State, Nigeria. They weighed 120- 180 g. The rats were housed in a standard environmental condition of temperature (30 ± 1 °C), humidity (60 ± 0.2 %) and 12 hours light and 12 hours dark cycle. They were fed with a formulation of high energy diet made from normal pellet diet powdered, mixed with sucrose, lard, vitamin-mineral mix and NaCl. All experimental procedures were approved by the Institutional Health Research Ethics Committee at the Osun State University, Osogbo, Nigeria (UNIOSUN/HREC/2012/A/002).

Plant Extract Preparation

The individual plant leaves were air-dried for 3 to 4 weeks under shade at room temperature. They were then pulverized using a motorized pulverizer yielding powdery products. 100 g dry powder of each sample were macerated in 1000 ml of 95 % ethanol and left for 48 hrs. The mixture was sieved using Whatmann filter paper 1, then the resulting filtrates were concentrated using rotary evaporator (LIDAi.DNA XMT – J7000/RE52-3).

Induction of Type I Diabetes Mellitus

This was carried out following an earlier protocol described by Ajayi *et al.* (2015) with slight modifications. Briefly, rats were subjected to diet manipulation with high energy diet for 2 months. Thereafter, they were given fat emulsion at 10ml/kg and fasted for 16 hours prior to i.p administration of alloxan (150 mg/kg BW/day) dissolved in 0.9 % saline. Blood glucose estimation was done by measuring the fasting blood glucose concentration at the beginning of the experiment and after 7 days of alloxan administration by standard spectrophotometry using Randox® kits (England) following manufacturer's instructions, and absorbance (505 nm) were read in duplicates using UV-Vis spectrophotometer (Jenway 6100, Dunmow, Essex, UK). Animals with blood glucose levels more than 200 mg/dL were used for the experiment.

Infliction of Wound

16 diabetic rats were allocated to three intervention groups of four rats per group. Wound was made on the back of each rat by excision according to the method of Abu-Al-Basal (2001), with slight modifications. Briefly, the back of each animal was shaved with hair clipper and sterilized with 70 % ethanol before 2 by 2 cm wound was made by lancet knife on a pre-determined area. The wound was left undressed, and no local or systemic antimicrobial agent was administered.

Extract Preparation for Topical Application

10 g of each extract was reconstituted in 100 ml of DMSO and used for topical application. The extract preparation was administered topically twice daily for 21 days. Wound in Group I animals were treated with topical application of povidone iodine, Groups II and III received a treatment of the respective traditional Africa herbal extracts, while Group IV was treated with DMSO as follows:

Group 1 (Control) - Diabetic rats treated with 3 ml of povidone iodine

Group 2 – Diabetic rats treated with 3 ml of *Nauclea latifolia* leave extract

Group 3 - Diabetic rats treated with 3 ml of *Manihot esculenta* leave extract

Group 4- Diabetic rats treated with 3 ml of DMSO.

A progressive decrease in the wound area was monitored periodically at 7 days interval measuring the wound contraction with tracing paper on the wound margin. The percentage reduction was calculated and granulation tissues were removed on the 7th, 14th and 21st post wound days and analyzed for collagen content.

Reagents and Chemicals

All reagents and chemicals used were of analytical grade and obtained from commercial sources.

Composition of High Energy Diet

43.9 % Normal Pellet Diet, 20 % Sucrose, 30 % Lard 6 % Vitamin-Mineral Mix, 0.1 % NaCl.

Statistical Analyses

All experimental readings were carried out in duplicates, using ANOVA (SigmaStat® 3.5) at $p < 0.05$ to imply statistical significance.

Determination of Total Phenolic Contents

The amount of total phenolics in extract was determined with Folin– Ciocalteu reagent according to the method of Singleton and Rossi (1965) with slight modification using tannic acid as a standard. Briefly, 1.0 ml of extract solution (5 mg/ml) was added in a 100 ml volumetric flask that contained about 60 ml distilled water. Then, 5.0 ml of Folin–Ciocalteu reagent was added and the content of the flask was mixed thoroughly.

After 1 - 8 min, 15.0 ml Na₂CO₃ (20 %) was added and the volume was made up to 100 ml using distilled water. The mixture was allowed to stand for 2 hr with intermittent shaking. The absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Jenway 6100, Dunmow, Essex, UK). The total phenolic content was determined as milligrams of gallic acid equivalent (GAE) using an equation obtained from the standard gallic acid calibration graph.

$$\text{Total Phenolic Content} = \frac{\text{Absorbance reading for test} \times \text{Standard concentration}}{\text{Absorbance reading for standard}}$$

Total Monomeric Anthocyanin by the pH-Differential Method

The total monomeric anthocyanin content was determined by the pH-differential method as described by Lee *et al.* (2005). Briefly, 0.1 mg of extract was dissolved in 10 ml DMSO was prepared as stock. The appropriate dilution factor for the sample was determined by diluting with 0.025 M KCl buffer (pH 1.0) at 700 nm (the absorbance was less than 1.2). The final volume of the sample was divided by the initial volume to obtain the dilution factor. Two dilutions of the sample were prepared, one with KCl buffer (pH 1.0) and the other with 0.4 M sodium acetate buffer (pH 4.5). These dilutions were allowed to equilibrate for 15 min. The absorbance of each dilution was measured at 700 nm (to correct for haze), against a blank cell filled with DMSO. The pigment content was calculated as malvidin-3-glucoside (M3G, molar extinction coefficient (ϵ) of 28,000 L.cm⁻¹.mol⁻¹ and molecular weight of 463.3 g).

$$\text{Absorbance of the diluted sample (A)} = (A_{700} \text{ pH 1.0} - A_{700} \text{ pH 4.5})$$

$$\text{Monomeric anthocyanin pigment (mg/l)} = (A \times \text{MW} \times \text{DF} \times 1000) \div \epsilon \times \text{Path length of cuvette}$$

Quantification of Collagen in Scar Tissues

To 1 ml of test sample (scar tissue homogenized in DMSO) was added 1 ml of freshly prepared 0.05 M CuSO₄ and 1 ml of 2.5 N NaOH and mixed by gentle swirling. The tubes were placed in a water bath at 40°C for 3-5 minutes. 1 ml of freshly prepared 6% H₂O₂ was added and the solution mixed gently and kept for another 10 minutes in the water bath with continuous shaking. Upon cooling, 4 ml of H₂SO₄ and 2 ml of 5% p-DMBA solution were added and content mixed, and further kept in water bath at 70°C for 16 minutes. After cooling to room temperature, the absorbance was measured against blank at 555 nm (Yura and Vogel, 1959).

Results

Percentage wound closure at the end of 21 days was found to be 100 % (Povidone Iodine), 98.75% (*Nauclea latifolia* leaf), 69.54 % (*Manihot esculenta* leaf), and 95.50% (DMSO). The ethanol extract of *Nauclea latifolia* leaf was found to contain alkaloids, saponins, anthocyanin, and phenols. Collagen content in *Nauclea latifolia* leaf-treated group, quantified three times at 7-day intervals was 2694.52 mg, 1526.10 mg and 1298.64 mg respectively; while that in *Manihot esculenta* leaf were 2047.43 mg, 1359.99 mg and 1078.64 mg respectively. The crude leaf extract of *Nauclea latifolia* showed promising potential for wound healing. This may be due to its significantly higher phenolic acid and anthocyanin contents (35.45mg GAE/g DW and 1.638 mg/l, respectively) compared to contents when compared to *Manihot esculenta* (28.95mg GAE/g DW and 0.91 mg/l, respectively). While povidone Iodine proved to be the most effective, *Nauclea latifolia* leaf extract was significantly more potent than *Manihot esculenta* leaf extract; which in turn was significantly less potent than DMSO at $p > 0.05$.

Table 1: Presence of phytochemicals in ethanol extracts of *Nauclea alatifolia* and *Manihot esculenta* leaves

Phytochemicals	Ethanol extract of <i>N. latifolia</i> leaves	Ethanol extract of <i>M. esculenta</i> leaves
Tanins	+	+
Flavonoids	+	+
Saponins	+	+
Alkaloids	+	+

Table 2: Total Phenolic and Anthocyanin contents of ethanol extracts of *Nauclea alatifolia* and *Manihot esculenta* leaves

Quantification	Ethanol extract of <i>Nauclea latifolia</i> leaves	Ethanol extract of <i>Manihot esculenta</i> leaves
Total Phenolic Content (mg GAE/g DW)	35.45± 0.74 ^a	28.95± 1.41
Anthocyanin Content (mg M3G/l)	1.64± 0.012 ^a	0.91± 0.14

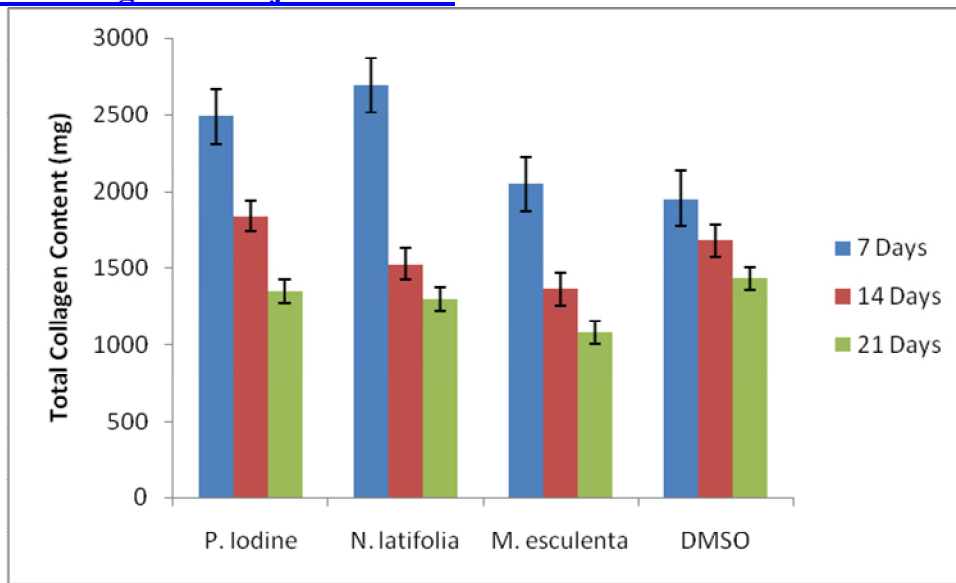


Figure 1: Total collagen content in scar tissues of wounded diabetic rats treated by topical application of crude extracts of *N. latifolia* and *M. esculenta* leaves

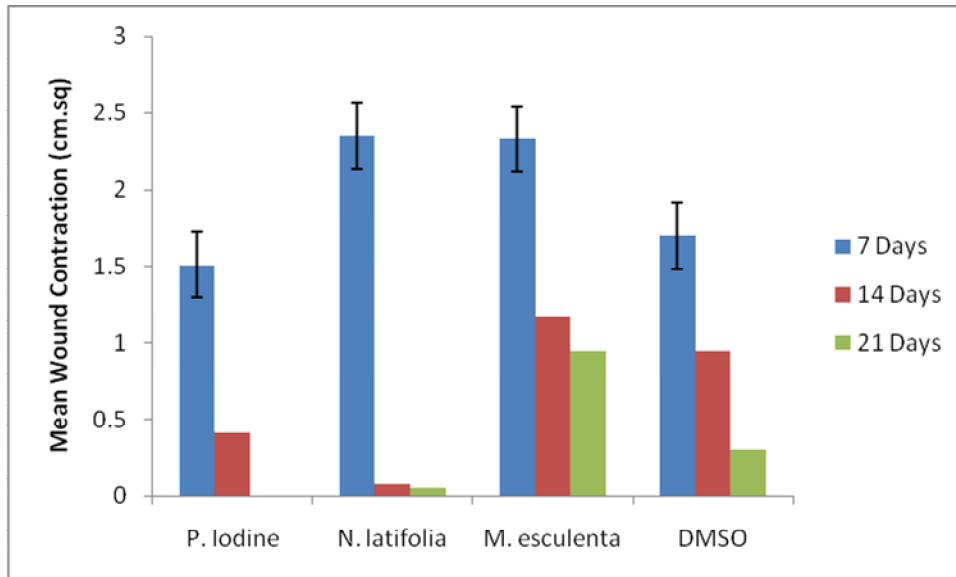


Figure 2: Mean wound contraction of wound of diabetic rats treated by topical application of crude extracts of *N. latifolia* and *M. esculenta* leaves

Discussion

The presence of the secondary metabolites tannins, flavonoids, saponins and alkaloids in the crude ethanol extracts of *Nauclea latifolia* and *Manihot esculenta* leaves confirms that the leaves of these plants are natural product sources of phytochemicals of medicinal importance. The four phytochemicals found to be present in the leaves used for this research have been reported to possess medicinal properties including wound healing potential (Agyare *et al.*, 2013). The collagen content of the scar tissues as well as the progression of wound contraction monitored over the period of 21 days indicated that the leaf extract of *M. esculenta* did not efficiently heal topical wound as much that of *N. latifolia*, which compared favourably with the positive standard, povidone iodine. Scar tissues recovered from the wound areas showed improved collagenation, re-epithelialization as well as rapid granulation compared to the vehicle control group.

Conclusion

Diabetes, if untreated, can lead to various complications such as diabetic foot ulcers, poor wound healing and infections. These adversely affect the quality of life of the patient as wound infection is a major complication in diabetic patients. Alternative therapies using natural products are coming to the fore as scientific evidences are on the increase in support of the efficacies of flavonoid-rich African traditional herbs in the management of diabetic complications. Many secondary plant metabolites have been shown to possess wound healing activities, and can synergistically improve wound in diabetes. The ethanol crude extract of *Nauclea latifolia* leaves has proven to be more potent than that of *Manihot esculenta* in wound healing in Type I diabetic rats. It stimulated wound contraction and collagen formation making it a promising natural product for further screening in search of new chemical entities that can be useful in diabetic wound management. Further structural elucidation, screening for short peptides as well as whole plant metabolomics is encouraged for future research on these plants.

Competing interests: The authors declare that they do not have any conflict of interest or competing / financial interests.

Authors' Contributions: Conceived and designed the experiments: AEIO. Supervised the experiments: AEIO. Performed the experiments: PG, OE. Analyzed the data: AEIO, PG, OE. Wrote the paper: AEIO.

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