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Original Article

Consumption of Cod Liver Oil-enriched *Vernonia amygdalina* Leaf-based Diet Promoted Wound Healing in Wound-inflicted Type 2 Diabetic Wistar Rats

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ABSTRACT

Background. *Diabetes mellitus is an important factor that contribute to non-healing chronic wound and 2 out of 10 diabetic patients in Nigeria present with diabetic foot ulcer. The aim of this study was to evaluate the effect of cod liver oil-enriched Vernonia amygdalina leaf-based diet (CLVA) on wound healing in wound-inflicted type 2 diabetic rats.* ***Methods****. Thirty-six albino rats were randomly assigned into 6 groups namely; control (C), diabetic untreated control (DC), reference drug control (RD), 10% CLVA, 20% CLVA and 30% CLVA. All the groups except C were diabetic rats inflicted with wound, while C were non diabetic rats inflicted with wound. Groups C, DC and RD were fed diet without cod liver oil (CLO) and Vernonia amygdalina (VA) leaves. The last 3 groups were fed 10, 20 and 30 % inclusion VA leaves and CLO in their diet. Feeding was done ad libitum for 14 days. Wound areas images, fasting blood glucose (FBG), Serum insulin, nitric oxide (NO) concentrations, wound contraction rate, interleukins-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor-kappa B (NF-κB) and vascular endothelial growth factor (VEGF) were monitored.* ***Results****. Results showed significant reduction ($p < 0.05$) in FBG, insulin, NO, IL-1β, IL-6 and TNF-α concentrations of rats fed on 10, 20 and 30 % to DC, while VEGF increased significantly ($p < 0.05$). Expression of iNOS, COX-2 and NF-κB were down-regulated in rats fed on all CLVA inclusion levels. Wound contraction rate increased significantly ($p < 0.05$) at the various inclusion levels compared to DC, with wound area images showing progressive wound closure in CLVA-fed groups.* ***Conclusion****. Consumption of CLVA promoted wound healing in wound-inflicted diabetic rats.*

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INTRODUCTION

Wound healing is a complex process in which the skin and the tissues under it repair themselves after injury [1]. In undamaged skin, the epidermis and dermis form a protective barrier against the external environment. When this barrier is broken, a regulated sequence of biochemical events is set into motion to repair the damage. This process is divided into different phases namely; blood clotting (hemostasis), inflammation, tissue growth (proliferation), and tissue remodeling (maturation) [1, 2]. The wound healing process however, is susceptible to interruption or failure, leading to the formation of non-healing chronic wounds. Among the early events of a wound-healing response is infiltration of inflammatory cells at the wound site [3]. However, prolonged inflammatory responses in wounds are associated with impaired healing [4, 5].

An important factor that contributes to non-healing chronic wound is diabetes mellitus [2]. With diabetes prevalence of 5.7%, Nigeria is currently home to about 5 million adults living with diabetes [6]. This appears to be a tip of the iceberg as it is estimated that about two-thirds of diabetes cases in Nigeria are yet undiagnosed [6]. Consequent upon this high prevalence of chronic undetected hyperglycemia, many individuals with diabetes present with already established chronic complications at the time of diagnosis [7]. One potentially preventable complication of diabetes that is associated with high morbidity and mortality is diabetic foot ulcer (DFU). It is estimated that a person with diabetes has up to 25% chance of developing DFU in his/her lifetime [8]. The burden of DFU is high in Africa [9], particularly in Nigeria [10, 11]. A recent update suggests that nearly 2 out of every 10 out-patients with diabetes in Nigeria have diabetic foot disease [11], and DFU accounts for nearly a third of diabetes-related hospital admissions [12]. Diabetic foot ulcer is associated with prolonged hospital stay, substantial economic burden and high mortality [13, 14]. Perhaps the most unpleasant potential consequence of DFU besides death is lower extremity amputation (LEA).

During impaired wound healing associated with diabetes, inflammatory response includes accumulation of macrophages which exhibit a persistent high levels of pro-inflammatory molecules like interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and the protease matrix metalloprotease-9 (MMP-9) [15, 16,17]. This pro-inflammatory phenotype may be induced by hyperglycemia [18, 19]. Therefore, persistent poorly controlled hyperglycemia in patients with diabetes mellitus may impair skin healing, so that even minor breaks in skin integrity can develop into deeper ulcers and easily become infected, particularly in the lower extremities [20].

Intensive control of plasma glucose however can prevent or delay this complication [21]. Many diabetic medications have demonstrated anti-inflammatory properties [22]. Since an increased inflammatory state is linked with impaired healing and diabetic medications may have anti-inflammatory properties, it seems reasonable to speculate that diabetic medications could have an influence on wound healing [22]. There have been various attempts to accelerate wound healing in diabetics, but so far only a few effective therapeutic remedies are available [23, 24]. Alternative therapeutic treatments using natural products are therefore highly demanded.

Vernonia amygdalina is a shrub or small tree of two to five meters in height. It is a member of the *Asteraceae* family and is popularly known as bitter leaf plant. The leaves are used traditionally in the preparation of Nigerian soups and porridges [25]. The antihyperglycemic potentials of *V. amygdalina* using different preparations of the plant had been reported by various researchers [25 - 29]. Cod liver oil on the other hand, is a dietary supplement derived from liver of cod fish (*Gadidae*) [30]. As with most fish oils, it contains omega-3 fatty acids namely; eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These omega-3 fatty acids found in fish oils are widely considered to benefit diseases related to chronic inflammation because of their anti-inflammatory properties [31]. Previous research suggested that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) significantly reduced the production of proinflammatory cytokines, which signal biological processes during the inflammatory stage of wound healing [31, 32].

The aim of this study therefore was to investigate the effect of consumption of cod liver oil enriched-*Vernonia amygdalina* leaf-based diet (CLVA) on wound healing in wound-inflicted type 2 diabetic rats.

METHODS

Ethical clearance for animal care and handling

Ethical clearance for animal care and handling for this study was obtained from Institutional Animal Care and Use Committee (IACUC) of the University of Ilorin in accordance with the recommendations for handling animals for research (approval number UERC/LSC 1001/2019).

Plant material and preparation

Fresh leaves of *Vernonia amygdalina* (VA) were collected in November, 2019. The plant was authenticated at the herbarium unit of the Department of Plant Biology, University of Ilorin, Nigeria and a voucher specimen number (UILH/001/2019/1023) was issued. Eight kilograms of VA leaves were washed and air dried to a constant weight of 6.8 kilograms. They were then pulverized into fine powder using an electronic laboratory blender and stored in an air tight container in a refrigerator (LG refrigerator, model No. GC131SL, China) at 4 ^{o}C.

Feed ingredients and preparation

Yellow corn, maize husk, soy bean oil, soy bean grain and sucrose were purchased in November, 2019 from *oja tutun*, a local market in Ilorin, Kwara State, Nigeria. The soy bean oil was a product of Sunola oil, Kewalram Nigeria Limited, Nigeria. Vitamin and mineral mix, DL-methionine and L- lysine were products of Rofat Feed Nigeria Limited, Ilorin, Nigeria.

Corn starch was prepared by rinsing and soaking 10 kg of yellow corn in 20 l of distilled water for 72 hours, followed by grinding and sieving. The filtrate was drained for 6 hours and oven dried at 40 ºC to constant weight. Ten kilograms of Maize husk was sun dried for 3 days and pulverised using commercial grinder. Soy bean grain (7 kg) was soaked in 15 l of distilled water for 6 hours and the seed coats were removed. Thereafter, it was sun dried for 3 days and ground to smooth texture. Corn starch, maize husk (cellulose source), sucrose, ground soybean, vitamin/mineral mix, DL- methionine and L-lysine were thoroughly mixed together in the various proportion indicated in Table 1. Soybean oil, cod liver oil and distilled water were added slowly to the mixed ingredients until the mixture became a paste. The paste was then grated on a wire mesh to form pellets which were oven dried at 40 ºC to a constant weight.

Table 1: Feed formulation for preparation of cod liver oil-enriched Vernonia amygdalina leaf-based diet

Ingredients g/kg	Control(C)	Diabetic group (DC)	Reference drug (RD)	10% CLVA	20% CLVA	30% CLVA
Corn starch	512	512	512	412	312	212
Cellulose	40	40	40	40	40	40
Sucrose	100	100	100	100	100	100
Soybean	250	250	250	250	250	250
Soybean Oil	40	40	40	30	20	10
Cod liver oil	-	-	-	10	20	30
Vitamin/mineral mix	50	50	50	50	50	50
D- Methionine	4	4	4	4	4	4
L-lysine	4	4	4	4	4	4
***V. amygdalina* leaves**	-	-	-	100	200	300
Total	1000	1000	1000	1000	1000	1000

CLVA – cod liver oil-enriched Vernonia amygdalina leaf-based diet

Animal care, grouping and treatment

Thirty-six Wistar rats of norvegicus strain with an average weight of 130 g ± 20 g were obtained from the Animal Holding Unit of the Department of Biochemistry, University of Ilorin, Nigeria. The rats were housed in well ventilated plastic cages (23.38×12.13×9.00 (Inches)) and allowed to acclimatize to animal house conditions; temperature (25 ± 2 °C), relative room humidity (55%) and alternating light-dark cycle (12 h/12 h), for 7 days. They were fed with normal rat pellet (product of premier feed mills, no 1, Eagle flour road, Premier Feeds Company Limited, Ibadan, Nigeria) and tap water *ad libitum* prior to the commencement of the experiment. The rats were randomly selected into 6 groups of 6 rats each and fed with experimental diets for 14 days as depicted in Table 2.

Table 2: Animal grouping and feed administration

S/No.	Groups	Description of treatments
1	Control rats (C)	Non-diabetic, wound-inflicted rats fed feed without cod liver oil and VA leaves
2	Diabetic control (DC)	Diabetic, wound-inflicted rats fed feed without cod liver oil and VA leaves
3	Reference drug (RD)	Diabetic, wound-inflicted rats administered Metformin (14.29 mg/kg b. wt.) and fed feed without cod liver oil and VA leaves
4	10 % cod liver oil-enriched *Vernonia amygdalina* leaf based-diet,	Diabetic, wound-inflicted rats fed feed with 10 ml cod liver oil and 100 g VA leaves
5	20 % cod liver oil-enriched *Vernonia amygdalina* leaf based-diet,	Diabetic, wound-inflicted rats fed feed with 20 ml cod liver oil and 200 g VA leaves
6	30 % cod liver oil-enriched *Vernonia amygdalina* leaf based-diet,	Diabetic, wound-inflicted rats fed feed with 30 ml cod liver oil and 300 g VA leaves

VA - Vernonia amygdalina; b.wt. – Body weight

Induction of diabetes mellitus

Rats in diabetic untreated group, reference drug, 10 %, 20 %, and 30 % CLVA groups were given free access to 10 % w/v fructose in water *ad libitum* for 2 weeks, while the control rats (non-diabetic rats) were given distilled water. At the end of 2 weeks administration of fructose solution, all the groups were fasted overnight and each of the fructose-fed rats was injected intraperitoneally with a freshly prepared 40 mg/kg b. wt. streptozotocin (STZ), (dissolved in 0.1 M citrate buffer, pH 4.5). A week after administration of STZ, FBG concentrations of the rats were determined using AccuChek active glucometer and strips by withdrawing blood from the rats' tail. Rats showing glucose concentration above 125 mg/dl were considered diabetic [33].

Infliction of wound

All rats were anaesthetized with dichloromethane by nasal inhalation following confirmation of diabetes mellitus. The dorsal fur of each rat was shaved with a razor blade and disinfected with 70 % v/v ethanol prior to wound incision. A standardized full thickness open excision wound with dimension 2 cm by 2 cm was then created using a sharp scalpel. The rats were placed back in their cages and administration of test diet commenced.

Animal sacrifice and collection of wound samples

Rats were sacrificed 24 hours after the last day of treatment (day 15). They were anaesthetized with dichloromethane and sacrificed by cutting the jugular vein using a sterile scalpel. Blood samples were collected into plain sample bottles for biochemical analysis. One gram of wound tissue from each rat was cut into pieces using a scalpel. Wound tissues were rinsed with phosphate buffer saline (PBS), homogenized in 1 ml of PBS and stored overnight at -20°C.

Determination of fasting blood glucose concentration and body weight of experimental rats

Fasting blood glucose (FBG) concentration of all the experimental rats was determined using Accu chek glucometer, Roche, Germany. Blood was withdrawn from the tail of each rat and dropped at the center of the green field on the strip already inserted into the glucometer. This was done before administration of STZ (day 0), after induction of diabetes was confirmed (Day 1) and then subsequently every other day for 14 days. Body weight of each rat was determined at 48 hours interval throughout the experiment using an electronic weighing scale (KERN (EMS 6K0.1), Tischwage, Germany).

Determination of serum insulin concentration

Serum insulin concentration was determined by day 15 (twenty-four hours after the last day of treatment), following manufacturer's instructions in insulin ELISA kit (product of Calbiotech Inc., 1935 Cordell Ct, El Cajon, CA 92020, USA). Absorbance was read at 450 nm using a multi plate ELISA reader (Biorad- 680, BIORAD Ltd., Japan).

Determination of wound contraction rate

The outer wound margins in this study were measured using a vernier calipers. The wound contraction rate was determined using the following formula [34]:

$$\text{Wound contraction rate (\%)} = \frac{Initial\ wound\ size - wound\ size\ on\ specific\ day\ (mm^2)}{Initial\ wound\ size\ (mm^2)} \times 100\ \%$$

Images of the wound contraction process were taken using a digital camera to assess wound closure on days 1, 7 and 14.

Determination of interleukin 1β, interleukin 6, tumor necrotic factor- α, inducible nitric oxide synthase, nuclear factor kappa B, cyclooxygenase 2 and vascular endothelial growth factor concentrations

The concentrations of proinflammatory cytokines (IL-6, IL-1 β, TNF-α), growth factor (VEGF) and mediators (iNOS, COX-2 activities and NF-kB concentration), were determined by following manufacturer's instruction using ELISA kits (Cusabio technology ELISA kits, Wuhan, China). Briefly, 100 µL of standard and sample were added to each well. The wells were then covered with adhesive strip and incubated for 2 hours at 37 ºC. Liquid was removed from each well and 50 µL of Biotin-antibody was then added to each well and covered with a new adhesive strip. Wells were then incubated for 1 hour at 37 ºC again. After incubation, each well was aspirated and washed by filling with 200 µL of wash buffer using a squirt bottle. Wells were then allowed to stand for 2 minutes and liquid completely removed. This process was repeated 2 times for a total of 3 washes. After the last wash, the plate was inverted and blotted against a clean paper towel. Horseradish peroxidase (HRP)-avidin (100 µL) was added to each well and the microtiter plate covered with a new strip and incubated for 1 hour at 37 °C. The aspiration and wash process was repeated 5 times as described previously. Ninety (90) µL of 3, 3',

5, 5'-Tetramethylbenzidine (TMB) substrate was thereafter added to each well and incubated for 15 minutes at 37 °C. Stop solution (50 µL) was added to each well and the plate tapped gently to ensure thorough mixing. The optical density of each well was determined using a microplate reader (Biorad- 680, Biorad Ltd., Japan) at 450 nm.

Measurement of nitric oxide concentration in wound lysate

Nitric oxide concentration was determined using colorimetric assay kit (Oxford Biomedical Research Inc., Oxford, MI 48371 U.S.A). The manufacturer's instruction was followed to carry out this assay. Basically, standards and samples (85 µL) were added to microplate in duplicate. Nitrate reductase (10 µL) was then added to each well, followed by addition of 10 µL of NADH working solution to each well. The plate was then shaken for 20 minutes at room temperature on a plate shaker. Colour reagent #1 (50 µL) was added to each well and shaken briefly, followed by the addition of colour reagent #2 (50 µL) to each well and subsequent shaking for 5 minutes at room temperature. Absorbance was measured at 540 nm using a microplate reader (Biorad- 680, BIORAD Ltd., Japan). NO concentration was extrapolated from the standard curve plotted.

Statistical analysis

Data were expressed as mean of 6 determinations ± standard error of mean (SEM). The data were subjected to statistical analysis using the IBM® statistical package for social sciences (SPSS) software version 20. All significant differences were determined by one way analysis of variance (ANOVA). Post hoc multiple comparisons were done using Duncan's multiple range test. The level of significance was set at $p < 0.05$ (confidence level = 95 %).

RESULTS

Fasting blood glucose concentration in wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet (CLVA)

There was a significant increase in blood glucose after STZ administration. However, following consumption of 10, 20 and 30 % CLVA, there was a significant decrease ($p<0.05$) in the blood glucose level of the CLVA-fed groups compared to the wound-inflicted diabetic untreated (DC) group. Amongst the various inclusion levels of CLVA, FBG of 20 % CLVA was not significantly different ($p>0.05$) to control group by day 9 of the experiment and by the 11th day of the experiment. There was no significant difference ($p>0.05$) in the blood glucose levels of both 10 and 30 % CLVA-fed rats compared with the control.

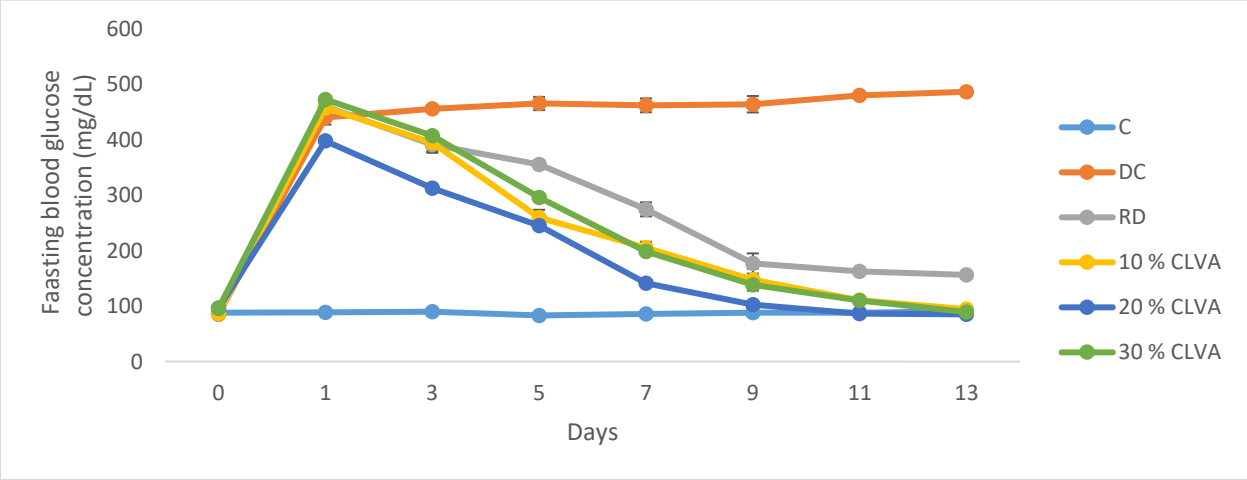

Figure 1: Fasting blood glucose of wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet

Values are expressed as mean of 6 determinations ± S.E.M

CLVA- cod liver oil-enriched Vernonia amygdalina leaf based-diet, C- control rats, DC - diabetic untreated rats, RD – reference drug treated rats

Day 0 = Fasting blood glucose concentration before diabetes induction

10 % CLVA - Diabetic, wound-inflicted rats fed feed with 10 ml cod liver oil and 100 g VA leaves

20 % CLVA - Diabetic, wound-inflicted rats fed feed with 20 ml cod liver oil and 200 g VA leaves
30 % CLVA - Diabetic, wound-inflicted rats fed feed with 30 ml cod liver oil and 300 g VA leaves

Serum insulin concentration in wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet

Insulin concentration in wound-inflicted diabetic rats fed CLVA decreased significantly ($p<0.05$) when compared to with rats in DC group. However, there was no significant difference ($p>0.05$) among the 10, 20 and 30 % CLVA-fed rats (Table 2).

Average body weight of wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet

There was a significant increase ($p<0.05$) in the body weight of experimental rats throughout the study period, except the reference drug-treated group (RD) which decreased by week 3 through week 6 of the experiment (Figure 3).

Table 3: Serum insulin concentration in wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet

Group	Insulin (µg/L)
C	0.17 ± 0.01^{a}
DC	0.51 ± 0.03^{b}
RD	0.34 ± 0.09^{c}
10 % CLVA	0.36 ± 0.04^{c}
20 % CLVA	0.35 ± 0.04^{c}
30 % CLVA	0.38 ± 0.03^{c}

Values are expressed as mean of 6 determinations ± S.E.M. and those with different superscripts down the column are statistically different ($p < 0.05$)
CLVA- cod liver oil-enriched Vernonia amygdalina leaf-based diet, C- control rats, DC - diabetic untreated rats, RD – reference drug treated rats
Day 0 = Fasting blood glucose concentration before diabetes induction
10 % CLVA - Diabetic, wound-inflicted rats fed feed with 10 ml cod liver oil and 100 g VA leaves
20 % CLVA - Diabetic, wound-inflicted rats fed feed with 20 ml cod liver oil and 200 g VA leaves
30 % CLVA - Diabetic, wound-inflicted rats fed feed with 30 ml cod liver oil and 300 g VA leaves

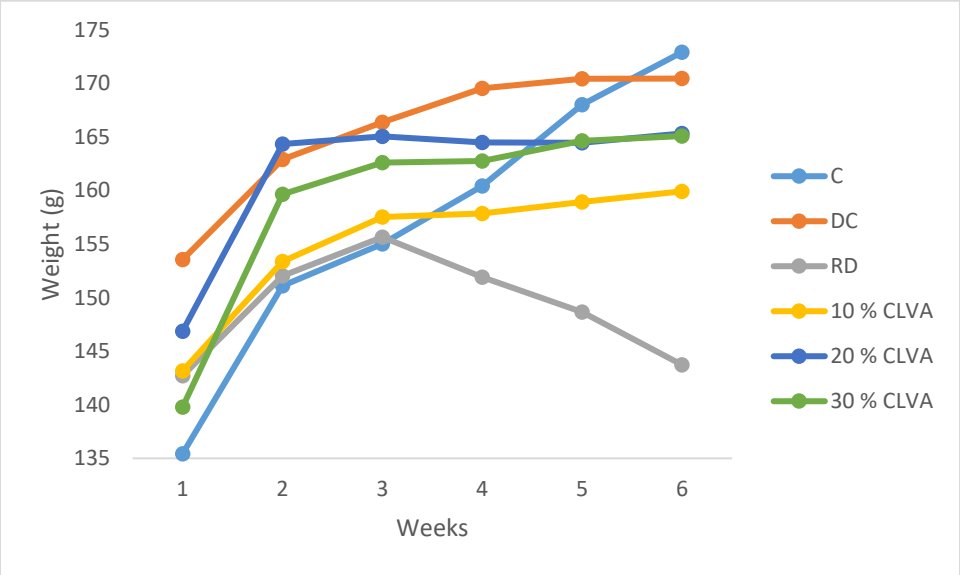

Figure 2: Body weight of wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet
Values are expressed as mean of 6 determinations ± S.E.M.
CLVA- cod liver oil-enriched Vernonia amygdalina leaf based-diet, C- control rats, DC - diabetic untreated rats, RD – reference drug treated rats
10 % CLVA - Diabetic, wound-inflicted rats fed feed with 10 ml cod liver oil and 100 g VA leaves
20 % CLVA - Diabetic, wound-inflicted rats fed feed with 20 ml cod liver oil and 200 g VA leaves
30 % CLVA - Diabetic, wound-inflicted rats fed feed with 30 ml cod liver oil and 300 g VA leaves

Wound contraction rate in wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet
There was a significant increase ($p<0.05$) in the wound contraction rate of the 10, 20 and 30 % CLVA-fed rats compared to DC for days 7 and 14. The wound contraction rates of the CLVA-fed rats by day 14 was not significantly different ($p>0.05$) compared to C. There was no significant difference ($p<0.05$) in the wound contraction rate of 10, 20 and 30 % CLVA-fed rats by days 7 and 14 (Table 4).
Images of wound areas of experimental rats showed progressive wound closure for the 10, 20 and 30 % CLVA-fed rats compared to DC by day 14 of the experiment (Figure 3).

Nitric oxide concentration in wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet
Nitric oxide concentrations of the 10, 20 and 30 % CLVA-fed rats decreased significantly ($p<0.05$) compared to the rats in DC group. However, 30 % CLVA-fed group was not significantly different ($p>0.05$) compared to the C (Table 5).

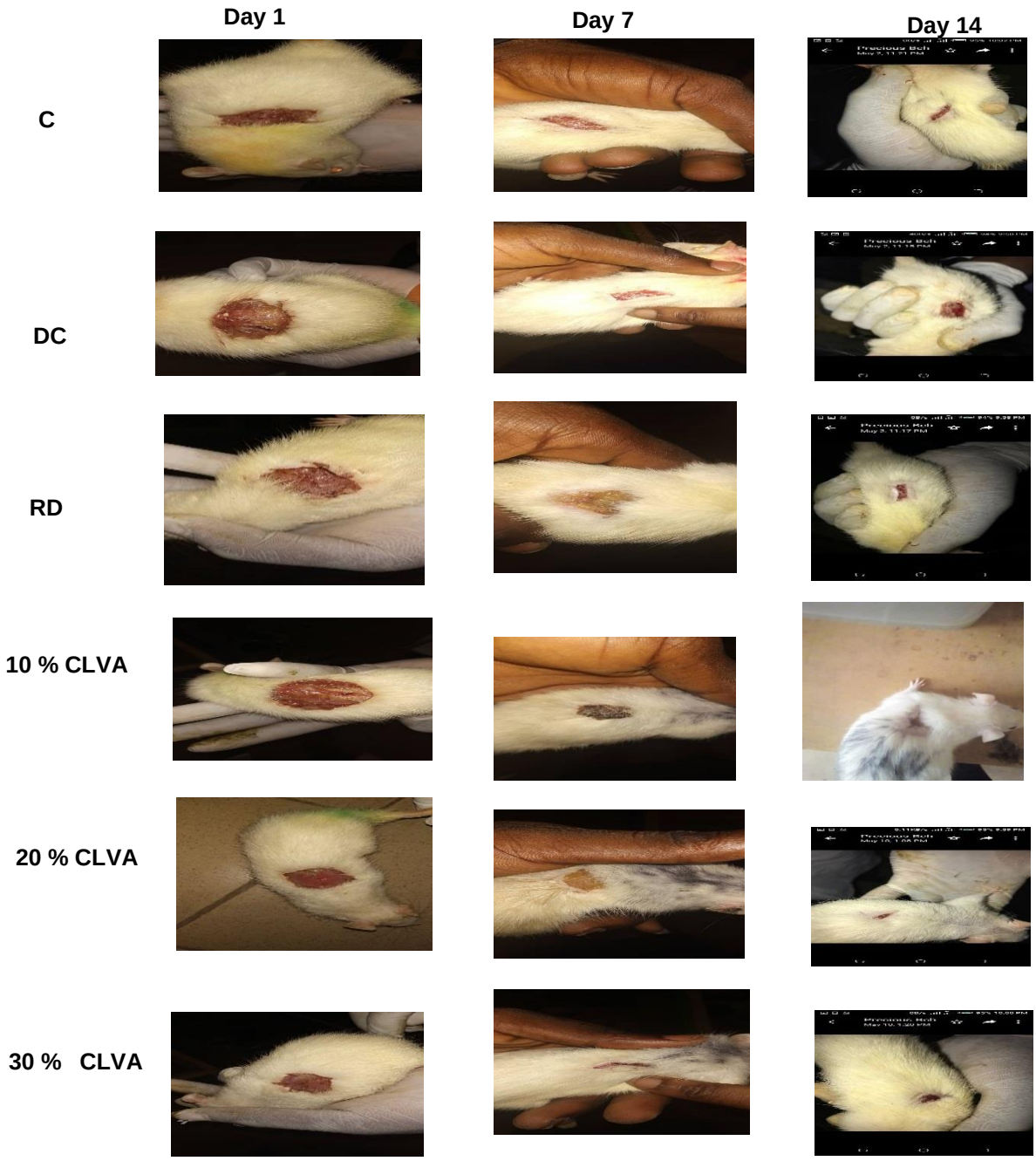

Figure 3: Images of wound in wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet

CLVA- cod liver oil-enriched Vernonia amygdalina leaf based-diet, C- control rats, DC - diabetic untreated rats, RD – reference drug treated rats

Day 0 = Fasting blood glucose concentration before diabetes induction

10 % CLVA - Diabetic, wound-inflicted rats fed feed with 10 ml cod liver oil and 100 g VA leaves

20 % CLVA - Diabetic, wound-inflicted rats fed feed with 20 ml cod liver oil and 200 g VA leaves

30 % CLVA - Diabetic, wound-inflicted rats fed feed with 30 ml cod liver oil and 300 g VA leaves

Table 4: Wound contraction rate in wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet

Groups	Wound contraction (%) Day 7	Day 14
C	26.33 ± 1.24^{a}	85.63 ± 4.00^{ac}
DC	11.46 ± 1.47^{b}	37.50 ± 0.22^{b}
RD	45.18 ± 1.27^{c}	86.17 ± 0.49^{ac}
10 % CLVA	50.33 ± 1.01^{d}	88.75 ± 0.23^{c}
20 % CLVA	47.83 ± 1.64^{cd}	77.21 ± 5.79^{a}
30 % CLVA	46.58 ± 1.18^{cd}	82.83 ± 3.80^{ac}

Values are expressed as mean of 6 determinations ± S.E.M. and those with different superscripts down the column are statistically different ($p < 0.05$)

CLVA- cod liver oil-enriched *Vernonia amygdalina* leaf-based diet, C- control rats, DC - diabetic untreated rats, RD – reference drug treated rats

Day 0 = Fasting blood glucose concentration before diabetes induction

10 % CLVA - Diabetic, wound-inflicted rats fed feed with 10 ml cod liver oil and 100 g VA leaves

20 % CLVA - Diabetic, wound-inflicted rats fed feed with 20 ml cod liver oil and 200 g VA leaves

30 % CLVA - Diabetic, wound-inflicted rats fed feed with 30 ml cod liver oil and 300 g VA leaves

Table 5: Nitric oxide concentration in wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf based-diet

Group	NO (µM)
C	14.17 ± 0.14a
DC	15.84 ± 018b
RD	15.29 ± 0.06c
10 % CLVA	12.03 ± 0.19d
20 % CLVA	12.96 ± 0.15e
30 % CLVA	14.24 ± 0.03a

Values are expressed as mean of 6 determinations ± S.E.M. and those with different superscripts down the column are statistically different ($p < 0.05$)

CLVA- cod liver oil-enriched Vernonia amygdalina leaf-based diet, C- control rats, DC - diabetic untreated rats, RD – reference drug treated rats, NO – Nitric oxide concentration

10 % CLVA - Diabetic, wound-inflicted rats fed feed with 10 ml cod liver oil and 100 g VA leaves

20 % CLVA - Diabetic, wound-inflicted rats fed feed with 20 ml cod liver oil and 200 g VA leaves

30 % CLVA - Diabetic, wound-inflicted rats fed feed with 30 ml cod liver oil and 300 g VA leaves

Proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and healing growth factor (VEGF) in wound tissues of wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet

As shown in Table 6, the concentrations of interleukin-1β, Interleukin-6 and tumor necrosis factor- α in wound lysates of 10, 20 and 30 % CLVA-fed rats were significantly reduced ($p<0.05$) compared to DC. However, TNF-α concentrations for the 20 and 30 % CLVA-fed rats were not significantly different ($p>0.05$) to control. The concentrations of vascular endothelial growth factor in wound tissues of wound-inflicted diabetic rats fed cod liver oil-enriched *Vernonia amygdalina* leaf-based diet at 10, 20 and 30 % inclusion levels were significantly increased ($p<0.05$) compared to DC (Table 6). Amongst the various inclusion levels, the VEGF concentration of 30 % CLVA-fed group reduced significantly ($p<0.05$) compared to 10 and 20 % CLVA-fed groups.

Wound mediators (iNOS, COX-2 and NF-κB) in wound tissues of wound-inflicted diabetic rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet

The expression of iNOS, COX-2 and NF-κB were down-regulated in significantly ($p<0.05$) 10, 20 and 30 % CLVA-fed wound-inflicted diabetic rats compared to DC (Table 7). There was no significant difference ($p>0.05$) in the expression of COX-2 and iNOS in RD, 10, 20 30 % CLVA-fed rats compared to rats in control group (C).

Table 6: Selected proinflammatory cytokines concentration in wound tissues of rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet

Groups	IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	VEGF (ng/ml)
C	104.50 ± 0.34^{a}	15.17 ± 0.09^{a}	145.42 ± 2.66^{a}	19.47 ± 0.40^{a}
DC	362.74 ± 5.62^{b}	30.86 ± 0.47^{b}	268.99 ± 5.24^{b}	0.78 ± 0.06^{b}
RD	109.78 ± 0.92^{a}	18.37 ± 0.38^{c}	239.90 ± 6.99^{c}	1.69 ± 0.14^{c}
10 % CLVA	108.49 ± 1.06^{c}	24.21 ± 0.31^{d}	227.15 ± 2.82^{c}	8.96 ± 0.11^{c}
20 % CLVA	167.14 ± 1.60^{e}	29.44 ± 0.01^{e}	146.96 ± 3.93^{a}	8.01 ± 0.29^{d}
30 % CLVA	150.48 ± 3.51^{d}	27.52 ± 0.92^{f}	145.45 ± 0.18^{a}	4.69 ± 0.28^{e}

Values are expressed as mean of 6 determinations ± S.E.M. and those with different superscripts down the column are statistically different ($p<0.05$); C – Control, DC- diabetic control, RD- reference drug(metformin), CLVA- cod liver oil enriched Vernonia amygdalina leaf-based diet

IL-1β – Interleukin 1 β; IL-6 - Interleukin 6; TNF-α – Tumor necrosis factor α; VEGF – Vascular endothelial growth factor

10 % CLVA - Diabetic, wound-inflicted rats fed feed with 10 ml cod liver oil and 100 g VA leaves
20 % CLVA - Diabetic, wound-inflicted rats fed feed with 20 ml cod liver oil and 200 g VA leaves
30 % CLVA - Diabetic, wound-inflicted rats fed feed with 30 ml cod liver oil and 300 g VA leaves

Table 6: Selected wound mediators in wound tissues of rats fed cod liver oil-enriched Vernonia amygdalina leaf-based diet

Groups	NF-kB(pg/ml)	iNOS(IU/ml)	COX-2(ng/ml)
C	231.06 ± 2.42^{a}	11.49 ± 1.18^{a}	2.87 ± 0.37a
DC	424.18 ± 14.28^{b}	13.46 ± 0.06^{b}	5.78 ± 0.71^{b}
RD	419.54 ± 4.54^{b}	11.75 ± 0.03^{a}	3.59 ± 0.18^{a}
10 % CLVA	399.59 ± 8.44^{c}	12.22 ± 0.09^{a}	3.44 ± 0.41^{a}
20 % CLVA	407.61 ± 4.00^{c}	11.78 ± 0.06^{a}	3.43 ± 0.21^{a}
30 % CLVA	356.78 ± 5.96^{d}	11.84 ± 0.02^{a}	3.39 ± 0.14^{a}

Values are expressed as mean of 6 determinations ± S.E.M. and those with different superscripts down the column are statistically different ($p<0.05$); C – Control, DC- diabetic control, RD- reference drug(metformin), CLVA- cod liver oil enriched Vernonia amygdalina leaf-based diet

iNos – Inducible nitric oxide synthase; COX-2 – cyclooxygenase; NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells.

10 % CLVA - Diabetic, wound-inflicted rats fed feed with 10 ml cod liver oil and 100 g VA leaves
20 % CLVA - Diabetic, wound-inflicted rats fed feed with 20 ml cod liver oil and 200 g VA leaves
30 % CLVA - Diabetic, wound-inflicted rats fed feed with 30 ml cod liver oil and 300 g VA leaves

DISCUSSION

Several reviews and studies have shown that the two major agents in our formulated diet; *Vernonia. amygdalina* (VA) leaves and cod liver oil (CLO) contain several secondary metabolites namely; flavonoids, saponins, alkaloids, tannins, phenolics, terpenes, steroidal glycosides, triterpenoids, and sesquiterpene lactones for VA [35-41] and omega-3 fatty acids; eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for CLO. All of these compounds gave VA and CLO their different pharmacological properties such as anti-diabetic and anti-inflammatory, amongst others [42-45]. In this study, it was hypothesized that consumption of CLVA would promote wound healing in wound-inflicted diabetic rats.

Induction of type 2 diabetes mellitus was successfully induced in the experimental rats as confirmed by the increase in the fasting blood glucose of the fructose and streptozotocin (STZ) administered rats (Figure 1) facilitated by the administration of fructose and STZ. STZ is a diabetogenic agent [46] which act by accumulating selectively in the pancreatic β-cell through the low-affinity glucose transporter 2 (GLUT2) in the plasma membrane, where it causes β-cell death by deoxyribonucleic acid (DNA) fragmentation [46]. Pancreatic β-cell death ultimately leads to deficiency or inadequate secretion of insulin [46, 47], the hormone that facilitate blood glucose utilization at the peripheral tissues, resulting in hyperglycemia as recorded in this study. Inducing diabetes mellitus successfully in an animal model is important in order to have an appropriate tool for diabetes related research and the choice of rodent is considered because it is relatively cheap and they are anatomically, physiologically and genetically similar to humans [48, 49].

Consumption of CLVA at the various inclusion levels by the experimental rats, ameliorated the hyperglycemia induced by STZ to normoglycemia. The antihyperglycemic activities of some of the constituents of *Vernonia amygdalina (*VA) have been earlier reported [29,50], which might be acting by inhibiting the activities of α-amylase and α-glucosidase thereby preventing the absorption of glucose in the circulatory system [42-44]. Therefore, CLVA could be a potent and safer alternative to orthodox medications in the management of hyperglycemia which characterize type 2 diabetes mellitus. In view of the importance of CLVA in the management of wound healing in a diabetic state, it is important to control hyperglycemia to normal levels so as to facilitate wound healing [51,52].

Among the various inclusion levels of CLVA investigated, the 20 % CLVA reduced the FBG levels rapidly than the 10 and 30 % CLVA, having achieved normoglycemia in the wound-Inflicted diabetic groups by day 9 of the experiment, while the other two inclusion levels produced normoglycemia by day 11 of the experiment. The reason for this variation cannot be clearly explained in this study.

Similarly, consumption of the various inclusion levels of CLVA was able to decrease the elevated level of insulin concentration observed in this study. Hyperinsulinemia is most often associated to insulin resistance [53], a condition that characterize type 2 diabetes mellitus. This is because the peripheral tissues have lost sensitivity to the action of insulin in type 2 diabetes, which results in elevation of insulin in the circulatory system in an untreated state. The constituents of CLVA were able to improve insulin sensitivity at the peripheral tissues at end of the study period. This might also be a possible mechanism involved in the amelioration of hyperglycemia earlier reported, but unlike results obtained for blood glucose level, there was no variation in the results of insulin concentrations among the various inclusion levels. The initial variation among the 10, 20 and 30 % CLVA-fed rats however could not be explained.

Growth is a key indicator of dietary adequacy, which is monitored in relation to expected pattern of weight gain in animals [54]. The observed weight gain in the CLVA-fed rats at the various inclusion levels suggest that VA leaves and CLO did not alter the dietary adequacy of the formulated diet.

The increased concentrations of interleukin-1β, Interleukin-6 and tumor necrosis factor-α recorded in the untreated diabetic wound-inflicted rats suggests impairment in wound recovery pathway. This may be as a result of the persistent hyperglycemia reported in that group. Normally, tissue injury induces release of proinflammatory cytokines (IL-6, IL-1β and TNF-α) from tissue resident macrophages, however prolonged release of these cytokines results in delay recovery of damaged tissues [55]. Diabetes is a major factor that contributes to delay in wound recovery because the presence of high concentration of glucose at wound site promotes microbial growth and leads to inflammatory signaling activation [1]. Both VA and CLO utilized in the formulation of CLVA in this study have been reported to possess anti-inflammatory property [56, 57]. This property was demonstrated in this study by the reduction in the concentrations of the proinflammatory cytokines, which was aided by the antihyperglycemic activity of VA. The constituents of VA and CLO might possess the ability to inhibit the release of TNF-α. TNF amplifies and prolongs inflammatory response by activating other cells to release IL-1. Following the inflammatory phase in wound healing is the proliferative and remodeling phases. Another mechanism of wound healing is increase production of vascular endothelial growth factor (VEGF) which was recorded in this study in the CLVA-fed rats. VEGF stimulates wound healing through angiogenesis but likely promotes collagen deposition and epithelialization as well [58].

Further, expressions of iNOS, COX-2 and NF-κB were down-regulated in the wound tissues of CLVA-fed rats (Table 6). Down-regulation of the expression of iNOS in the CLVA-fed rats brought about a concomitant decrease in the production of NO as well (Table 4). This suggest that CLVA might also possess the ability to inhibit iNOS activity resulting in decreased synthesis of NO. NO is a highly reactive free radical gas that acts as a messenger molecule for discrete physiological responses. It is toxic to bacteria and produces a cytotoxic wound environment; it is a potent vasodilator, increases vascular permeability, and could inhibit platelet aggregation [59]. Abnormally high levels of NO in the wound environment have been shown to have detrimental effects on wound healing resulting in impaired wound healing [59]. COX-2 on the other

hand, is a critical mediator of inflammatory response. It functions by producing prostaglandins that control many aspects of the resulting inflammation which includes; induction of vascular permeability, infiltration and activation of inflammatory cells [60, 61]. The down regulation of COX-2 in the CLVA-fed rats might be attributed to the anti-inflammatory capacity of VA and CLO. During wound healing process, release of IL1-1 IL-6 and TNF-α leads to the expression of COX-2 which catalyses arachidonic acid to prostaglandins, prostacyclins and thromboxanes [62,63]. Prostacyclin and thromboxane A2 cause vasoconstriction necessary for hemostasis and prostaglandins (E and I series) contribute to inflammation by increasing vascular permeability and stimulating inflammatory cells [64,65]. The overexpression of cox-2 the activation of metalloproteinases resulting in direct destruction of extracellular matrix [66] and its overexpression contributes to wounds that do heal on time. Hence, CLVA might be promoting wound healing in the experimental rats by down regulating the expression of COX-2. NF-κB on the other hand, also serve as a mediator of inflammatory responses. It is a key transcription factor of M1 macrophages and is required for induction of a large number of inflammatory genes including those encoding for TNF-α, IL-1β, IL-6 and COX-2 [67]. It's down regulation as observed in this study by in the CLVA-fed groups would regulate the release of the proinflammatory cytokines and ultimately results in promotion of wound healing.

The final sign of the proliferation phase is wound contraction, which normally starts 5 days after injury. Wound contraction is a dynamic process through which connective tissue matrix is formed by collagen fibers synthetized by newly migrated fibroblasts. Then, fibroblasts differentiate to myofibroblasts that are responsible for tensile force to pull the wound edges toward the wound center, which results in gradually reducing the wound area [68]. This process was accelerated in the CLVA-fed groups as observed in this study. CLVA might be facilitating this process by promoting the synthesis of extracellular matrix components. Overall, wound closure was accelerated in the CLVA-fed groups especially, the 20 % fed groups,

CONCLUSION

Consumption of the various proportions of CLVA evaluated in this study for 14 days promoted wound healing in wound-inflicted diabetic rats by down regulating the expression of iNOS, COX-2 and NF-kB pathway, as well as promoting the synthesis of VEGF. However, inclusion levels of 20 and 30 % CLVA gave a better wound healing activity than the 10 % inclusion level. Therefore, incorporation of *Vernonia amygdalina* leaves and cod liver oil in indigenous diets might be beneficial to type 2 diabetic patients suffering from delayed chronic wounds.

Disclaimer

The article has not been previously presented or published, and is not part of a thesis project.

Conflict of Interest

There are no financial, personal, or professional conflicts of interest to declare.

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