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ORIGINAL STUDY

Fish Oil and *Vernonia amygdalina* Leaves-supplemented Biscuits Increased Insulin Secretion and Suppressed Pro-inflammatory Cytokines in Type 2 Diabetic Wistar Rats

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Abstract

This research determined the effect of fish oil *and Vernonia amygdalina*-enriched biscuits (FVSB) on insulin sensitivity and inflammatory cytokines in type 2 diabetic rats. Forty-nine male rats were grouped into seven, i.e. A-G. Type 2 diabetes (T2DM) was induced in groups B-G by administering 230 mg/kg body weight [BW] NAD (ip) and 65 mg/kg BW streptozotocin (ip). Group A represented the control (Non-diabetic + unsupplemented biscuit), B (untreated diabetic), C (diabetic + 120 mg/kg BW metformin), D-F (diabetic + FVSB at 1 g, 2 g and 3 g leaf inclusion respectively), and G (diabetic + conventional biscuit). The experiment lasted 35 days, and rats were monitored for changes in fasting blood glucose (FBG), insulin levels, interleukin-6 (IL-6), C-reactive protein (CRP), and tumor necrosis factor-α (TNF-α). FVSB reduced FBG, insulin, HOMA-IR, IL-6, CRP and TNF-α but increased HOMA-β score in comparison with untreated diabetic groups. FVSB reduced FBG in an exposure duration-dependent manner. At 3 g leaf inclusion level, FVSB reduced insulin and HOMA-IR to levels comparable to metformin. At 2 g leaf inclusion, FVSB reduced IL-6 to levels comparable with the sham control. At all the leaf inclusion levels, FVSB reduced CRP and TNF-α, while also increasing HOMA-β score to levels comparable to either the sham control or the metformin-treated groups. In conclusion, FVSB elicited insulin sensitivity-improvement activity by reducing FBG and insulin concentrations and also anti-inflammatory properties by reducing IL-6, CRP and TNF-α in diabetic rats. The 3 g leaf inclusion biscuit is the most potent and may be considered for the management of T2DM.

Keywords: Type 2 diabetes mellitus, Interleukin-6, Insulin resistance, Cytokines, Metformin

1. Introduction

Type 2 diabetes mellitus (T2DM) is defined by a conjunction of peripheral insulin resistance and insufficient insulin production from pancreatic beta cells. Insulin resistance, caused by heightened concentrations of free fatty acids and proinflammatory cytokines in plasma, results in diminished glucose transport into muscle cells, increased hepatic glucose synthesis, and enhanced lipid catabolism [1].

Diabetes mellitus is a chronic metabolic disorder marked by hyperglycemia, which, if inadequately controlled, can result in damage to the heart, vasculature, eyes, kidneys, and nerves over time. More than 90 % of diabetes mellitus cases are classified as type 2 diabetes mellitus (T2DM). It is characterized by inadequate insulin synthesis from

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pancreatic islet β-cells and tissue insulin resistance (IR) [2,3].

A crucial element of T2DM is a persistent low-grade inflammatory condition. Diabetes mellitus induces heightened lipolysis, leading to hyperlipidemia and an accumulation of free fatty acids (FFAs). Elevated FFAs and hyperglycemia result in heightened activity of oxidative pathways resulting in increased reactive oxygen species (ROS) generation [4]. Oxidative stress can stimulate the immune system by activating redox-sensitive transcription factors, nuclear factor kappa B (NF-kB) [5], which contributes to the early phases of low-grade inflammation. Low-grade systemic inflammation is defined by a two to threefold elevation in systemic plasma levels of cytokines, including tumor necrosis factor (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP) [6].

Although there is proof that receiving the best diabetes care can lead to fewer complications and better financial results, this is frequently not the case [7–10]. During the 1990s, the share of primary care visits by diabetic patients using five or more prescription drugs rose from 18 % to 30 %. Furthermore, the number of visits involving more than one medication for blood sugar control grew from under 1 %–17 %. [11]. The utilization of oral hypoglycemic agents rose from 45 % to 53 %, while the prevalence of combination therapy involving insulin and an oral agent increased from 3 % to 11 % [11]. A study involving diabetes patients in primary care revealed that they were prescribed a diverse range of medication combinations for glycemic control, with the majority using up to two medications. An increased quantity of antidiabetic drugs correlates with suboptimal glucose regulation, highlighting the constraints of existing drug treatment. Twenty-five percent of patients are prescribed glycemic medications that may have contraindications [12].

Over time, there has been a growing interest in researching alternative medicines derived from natural sources to supplement traditional diabetes care practices. Among the numerous natural therapies, *Vernonia amygdalina*, also known as bitter leaf, and fish oil have sparked interest for their possible therapeutic effects in diabetes. *Vernonia amygdalina* is a plant indigenous to West Africa and portions of Asia. It has a long history of usage in traditional medicine to treat a variety of conditions, including diabetes. It is high in secondary metabolites such polyphenols, flavonoids, and alkaloids. *Vernonia amygdalina* has antioxidant, anti-inflammatory, and hypoglycemic effects, making it an attractive choice for diabetes treatment [13,14]. Fish oil, on the other hand, comes from oily fish like salmon, mackerel, and sardines. It is famous for having a lot of omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega-3 fatty acids reduce inflammation by changing immune reactions and the production of cytokines. This may help lower the low-grade inflammation that comes with diabetes mellitus [15].

Fish oil and *Vernonia amygdalina* have been extensively studied for their potential roles in the management of diabetes [13–15]. However, existing research has predominantly examined the effects of fish oil and *V. amygdalina* leaves in isolation with respect to type 2 diabetes management. To date, no studies have evaluated the combined use of fish oil and *V. amygdalina* leaves formulated as a therapeutic biscuit, except those conducted by our research team [14]. Our investigation into this novel combination aimed to elucidate potential synergistic effects and assess its viability as a functional food product for diabetes management. Developing a therapeutic biscuit as an alternative to conventional pharmacological treatments holds promise, as it may enhance patient compliance by presenting treatment in the familiar form of food, rather than as a lifelong medication regimen. Improved adherence is critical, given that non-compliance and therapy discontinuation remain leading contributors to poor disease control across populations.

2. Materials and methods

2.1. Plant preparation

Vernonia amygdalina leaves were collected from a garden on Ajowa Street, Sango, Ilorin Nigeria (8°31′37.12116″N, 4°35′19.21848″E). The leaves were identified and verified at Plant Biology Department, University of Ilorin, Kwara State, Nigeria and assigned a voucher number (UILH/001/1324/2023) for reference purpose. The harvested leaves were rinsed and air-dried at room temperature (28 °C) and subsequently ground using an electric blender.

2.2. Collection of fish and extraction of oil

Fish oil was obtained from the visceral tissues of *Heteroclarias* spp. catfish. The tissues were obtained from catfish market located on Unity Road in Ilorin, Nigeria, where they are manually excised by the fish sellers. Oil extraction from fish viscera was conducted using the method previously described [16]. The chopped viscera (1000 g) was mixed with distilled water (1000 mL) in a cooking bowl and boiled (100 °C) for a duration of 50 min. Following a

cooling period of 3 h, the oil that had risen to the surface was then separated from the mixture with the aid of a separating funnel. The transparent oil underwent a process of decantation, sieving, and heating to remove any residual moisture.

2.3. Preparation of experimental biscuits

Four biscuit samples were made: biscuit devoid of fish oil and *V. amygdalina* (C), biscuit containing fish oil and 1 g of *V. amygdalina* leaf (FVSB-1g), biscuit containing fish oil and 2 g of *V. amygdalina* leaf (FVSB-2g), and biscuit containing fish oil and 3 g of *V. amygdalina* leaf (FVSB-3g). Biscuits incorporated with fish oil and *Vernonia amygdalina* leaf were produced based on Whitley's recipe, with slight adjustments in ingredient proportions [17]. Briefly, to prepare the control biscuit, wheat flour (240 g), sweetener (15 g), salt (1 g), baking powder (4 g), butter (60 g), and milk (60 g) were manually mixed to create a dough. The dough underwent kneading, was rolled out, shaped into rounds, and baked at 175 °C for a duration of 15 min. The test biscuits were prepared using the ingredients described above with different levels of VA (1 g, 2 g, 3 g) inclusion, and butter was substituted for 69.5 g of fish oil. Following baking, the biscuits were allowed to cool at room temperature and subsequently stored in Ziplock bags.

The conventional biscuit, designated CB, was a commercially manufactured biscuit (Yale Foods Industrial LTD, Ibadan, Nigeria), acquired from a local vendor in Ilorin. The constituents of the conventional biscuits used as listed on the label claim are; whole wheat flour, vegetable fat, sugar, fat filled milk, inverted syrup, soya lecithin, edible salt, glucose syrup, leavening agent (sodium bicarbonate, malic acid, ammonium bicarbonate) and antioxidant (E321).

2.4. Experimental animals, diabetes induction and treatment administration

Forty nine (49) male Wistar rats of average weight 121.5 ± 10.5 g, which were obtained from the animal-rearing facility of the University of Ilorin, were utilized for this study. They were accommodated in adequately ventilated cages and permitted a 14-day period of acclimatization to animal house conditions (temperature -28–31 °C; a 12-h natural light/dark cycle; and humidity -50–51 %). Throughout the acclimatization phase, they were supplied with rat pellets (Premier Feeds Company Limited, Nigeria) and had unrestricted access to drinkable water.

Forty-nine male rats were distributed into seven groups, each containing seven rats (i.e. A-G). Type 2 diabetes (T2DM) was induced in groups B-G by intraperitoneal injection of nicotinamide adenine dinucleotide (NAD) in deionized water (230 mg/kg BW). Fifteen minutes later, intraperitoneal injection of 65 mg/kg BW streptozotocin freshly dissolved in 0.1 M ice-cold citrate buffer (pH 4.5) was administered to the rats. Blood glucose levels were measured after 72 h, and rats with levels exceeding 200 mg/dL were selected for further experimentation.

The seven groups (i.e. A-G) were treated as follows:

Group A (Sham Control): Non-diabetic + unsupplemented biscuit,

Group B (Untreated diabetic Control): NAD (230 mg/kg BW) + Streptozotocin (65 mg/kg BW) + unsupplemented biscuit,

Group C (Standard Drug Control): NAD (230 mg/kg BW) + Streptozotocin (65 mg/kg BW) + 120 mg/kg BW metformin

Group D: NAD (230 mg/kg BW) + Streptozotocin (65 mg/kg BW) + FVSB at 1 g leaf inclusion,

Group E: NAD (230 mg/kg BW) + Streptozotocin (65 mg/kg BW) + FVSB at 2 g leaf inclusion,

Group F: NAD (230 mg/kg BW) + Streptozotocin (65 mg/kg BW) + FVSB at 3 g leaf inclusion,

Group G: NAD (230 mg/kg BW) + Streptozotocin (65 mg/kg BW) + conventional biscuit

Treatment lasted for 35 days. FBG was determined every week. Rats were anesthetized with dichloromethane 24 h after the final day of treatment and euthanized via jugular vein incision. Venous blood was collected into plain (non-anticoagulant) tubes and left to clot at room temperature (27 °C). The resulting serum was subsequently separated and transferred into clean vials using a Pasteur pipette for biochemical analysis.

2.5. Biochemical assays

2.5.1. Determination of fasting blood glucose (FBG) concentration

The ACCU CHECK® glucometer was utilized to assess FBG concentrations across all experimental groups. Tail vein blood samples were obtained from the rats. Weekly assessments of FBG levels were conducted over the course of the study.

2.5.2. Assessment of serum insulin level

Serum insulin levels were quantified following the protocol outlined by the manufacturer of the insulin ELISA kit (Calbiotech Inc., El Cajon, CA, USA). Optical density was read at 450 nm using a

microplate ELISA reader (Model 680, Bio-Rad Laboratories, Japan).

2.5.3. Assessment of Homeostasis Model for Insulin Resistance and Homeostasis Model for Beta Scores

The previously described mathematical formula was utilized to compute the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and the Homeostasis Model Assessment for Beta Scores (HOMA-β scores) [18];

$$HOMA-IR=\left[Insulin\left(\frac{\mu U}{l}\right)\times Bloodglucose\left(\frac{mmol}{l}\right)\right] \div 22.5$$

$$HOMA-\beta=[20\times insulin\ (\mu U/l)] \div \left[Blood\ glucose(mmol/l)-3.5\right]$$

2.5.4. Determination of Interleukin-6, C-reactive protein, and Tumor Necrosis Factor-α concentrations

Cusabio Technology ELISA kits sourced from Wuhan, China, were employed to assess the concentrations of IL-6, CRP, and TNF-α in the serum of the rats. Standard and sample (100 μL) were added to each well, covered with an adhesive strip, and incubated for 2 h at 37 °C. Subsequently, the liquid was extracted from each well, and 50 μL of biotin-antibody was added, followed by the application of a new adhesive strip. The incubation was extended for an additional hour at 37 °C. Following incubation, each well was aspirated and rinsed with 200 μL of wash buffer. The wells were permitted to remain undisturbed for 2 min prior to the complete removal of the liquid. The process was conducted twice, resulting in a total of three washes. The plate was inverted and blotted with a clean paper towel following the final wash. One hundred microliters of horseradish peroxidase-avidin was added to each well, followed by covering the microtiter plate with a fresh strip and incubating it at 37 °C for 1 h. The aspiration and washing process was repeated five times. Subsequently, 90 μL of 3,3′,5,5′-Tetramethylbenzidine substrate was added to each well and incubated at 37 °C for 15 min. To halt the reaction, 50 μL of stop solution was introduced into each well, and the plate was gently tapped to ensure thorough mixing. The optical density of each well was then measured at 450 nm using a microplate reader

2.6. Statistical analysis

Statistical analysis was performed using SPSS software version 20. A one-way analysis of variance (ANOVA) was used to determine significant differences, followed by Duncan's multiple range test for post hoc multiple comparisons. The significance level was set at $p < 0.05$ with a 95 % confidence interval. Data were presented as the mean of seven determinations ± SEM

3. Results

3.1. Blood glucose

FBG level in NAD and streptozotocin-induced diabetic rats fed FVSB is presented in Table 1. Prior to diabetes induction, there was no significant difference ($p > 0.05$) in FBG level in all the groups.

After 1 week of diabetes induction, FBG concentration was significantly elevated ($p < 0.05$) in all the NAD and streptozotocin-exposed rats when compared with the sham control group. FBG level in the FVSB-exposed group did not significantly differ ($p > 0.05$) from those of diabetic untreated (group B) and conventional biscuit-exposed (group G) groups, but significantly lower ($p < 0.05$) than that of metformin-treated rats (group C) (Table 1).

After week 2, FBG level in the FVSB-treated rats was significantly lower ($p < 0.05$) than the diabetic untreated group (group B). There was no significant

Table 1. Fasting blood glucose level in NAD and streptozotocin-induced diabetic rats fed fish oil and Vernonia amygdalina*-supplemented biscuits.*

Groups	FBG (mg/dL)					
	Pre-induction	Week 1	Week 2	Week 3	Week 4	Week 5
A	81.57 ± 5.79^{a}	82.86 ± 6.27^{a}	83.83 ± 6.78^{a}	75.83 ± 6.04^{a}	76.83 ± 4.30^{a}	70.17 ± 3.23^{a}
B	77.43 ± 4.04^{a}	258.57 ± 4.49^{b}	266.71 ± 3.97^{b}	272.29 ± 8.82^{b}	295.71 ± 4.70^{b}	304.00 ± 3.75^{b}
C	82.43 ± 4.46^{a}	239.00 ± 8.07^{c}	237.00 ± 3.87^{c}	206.00 ± 4.04^{c}	186.67 ± 1.17^{c}	150.33 ± 0.33^{c}
D	81.57 ± 2.93^{a}	258.33 ± 6.56^{b}	208.50 ± 4.99^{d}	206.40 ± 5.55^{c}	177.60 ± 3.94^{d}	146.00 ± 1.94^{d}
E	83.86 ± 2.94^{a}	250.43 ± 6.68^{b}	222.50 ± 5.39^{cd}	201.17 ± 7.95^{c}	180.25 ± 2.76^{d}	134.40 ± 1.88^{e}
F	85.14 ± 6.23^{a}	252.57 ± 6.67^{b}	232.00 ± 5.62^{c}	202.20 ± 4.94^{c}	175.80 ± 3.68^{d}	124.40 ± 1.88^{f}
G	85.71 ± 1.66^{a}	258.29 ± 6.97^{b}	259.20 ± 8.12^{c}	249.67 ± 4.88^{c}	260.33 ± 4.67^{e}	229.50 ± 3.50^{g}

n = 7 ± SEM; mean values with different superscripted alphabets are significantly different ($p < 0.05$), A (Non-diabetic + unsupplemented biscuit), B (diabetic + unsupplemented biscuit), C (Diabetic + 120 mg/kg BW metformin), D (Diabetic + FVSB at 1 g leaf inclusion), E (Diabetic + FVSB at 2 g leaf inclusion), F (Diabetic + FVSB at 3 g leaf inclusion), G (Diabetic + conventional biscuit); FVSB (Fish oil *and Vernonia amygdalina*-supplemented biscuits).

difference ($p > 0.05$) in FBG level in the FVSB-treated groups (at 2 and 3 g leaf inclusion levels) and conventional biscuit-treated rats when compared with the metformin-treated rats. However, FVSB at 1 g leaf inclusion level significantly reduced ($p < 0.05$) FBG level than metformin (Table 1).

After week 3, FBG level in all the diabetic treated rats was significantly lower ($p < 0.05$) than in the diabetic untreated group. There was no significant difference ($p < 0.05$) in the FBG level of rats fed FVSB and conventional biscuits and those exposed to metformin (Table 1).

After week 4, as usual, FBG level in all the diabetic treated rats was significantly lower ($p < 0.05$) than in the diabetic untreated group. FBG level in all the treated rats was significantly lower ($p < 0.05$) than in the untreated group. FVSB-fed rats showed a significantly lower ($p < 0.05$) FBG than the metformin-exposed rats and those fed conventional biscuits (Table 1).

Similarly, FBG level in all the diabetic treated rats was significantly lower ($p < 0.05$) than in the diabetic untreated group after week 5. Rats fed FVSB had a significantly lower ($p < 0.05$) FBG level than those exposed to metformin and the conventional biscuit. FVSB reduced FBG in rats as leaf inclusion level increased (Table 1).

3.2. Insulin concentration, homeostatic model assessment for insulin resistance, and pancreatic beta-score of experimental rats

Insulin concentration, Homeostatic Model Assessment for Insulin Resistance and pancreatic β-cell score in NAD and streptozotocin-induced diabetic rats fed fish oil and Vernonia amygdalina-supplemented biscuits is presented in Table 2.

Insulin concentration significantly increased ($p < 0.05$) in untreated diabetic rats in comparison to the sham control group. Exposure to FVSB significantly reduced ($p < 0.05$) insulin concentration when compared with the diabetic untreated group. FVSB at 3 g leaf inclusion level reduced insulin concentration to a level significantly comparable ($p > 0.05$) to metformin. There was no significant difference ($p > 0.05$) in insulin level of rats fed FVSB at 1 and 2 g leaf inclusion level and rats fed conventional biscuit (Table 2).

HOMA-IR was significantly increased ($p < 0.05$) while HOMA β-score was significantly reduced ($p < 0.05$) in diabetic untreated rats when compared with the sham control group. HOMA-IR significantly reduced ($p < 0.05$) in rats exposed to FVSB and metformin when compared with the diabetic untreated group. Only rats fed FVSB at 3 g leaf inclusion level had HOMA-IR comparable ($p > 0.05$) with that of metformin-exposed rats. HOMA- β-score was significantly increased ($p < 0.05$) in rats exposed to FVSB and metformin when compared with diabetic untreated rats. There is no significant difference ($p > 0.05$) in HOMA- β-score of FVSB-fed rats (all leaf inclusion levels) and that of metformin-exposed rats.

Table 2. Insulin concentration, Homeostatic Model Assessment for Insulin Resistance and pancreatic β-cell score in NAD and streptozotocin-induced diabetic rats fed fish oil and Vernonia amygdalina-supplemented biscuits.

Groups	Insulin (mIU/L)	HOMA-IR	HOMA β-score
A	4.75 ± 0.28^{a}	0.86 ± 0.11^{a}	9.04 ± 1.15^{a}
B	6.26 ± 0.28^{b}	3.63 ± 0.12^{b}	0.46 ± 0.08^{b}
C	4.90 ± 0.07^{a}	2.36 ± 0.13^{c}	0.80 ± 0.12^{c}
D	5.45 ± 0.24^{c}	3.21 ± 0.06^{d}	0.73 ± 0.23^{c}
E	5.43 ± 0.35^{c}	3.14 ± 0.03^{d}	0.76 ± 0.04^{c}
F	4.72 ± 0.21^{a}	2.18 ± 0.28^{c}	0.97 ± 0.19^{c}
G	5.97 ± 0.06^{b}	3.33 ± 0.20^{b}	0.52 ± 0.08^{b}

n = 7 ± SEM; mean values with different superscripted alphabets are significantly different ($p < 0.05$), A (Non-diabetic + unsupplemented biscuit), B (diabetic + unsupplemented biscuit), C (Diabetic + 120 mg/kg BW metformin), D (Diabetic + FVSB at 1 g leaf inclusion), E (Diabetic + FVSB at 2 g leaf inclusion), F (Diabetic + FVSB at 3 g leaf inclusion), G (Diabetic + conventional biscuit); FVSB (Fish oil and Vernonia amygdalina-supplemented biscuits); HOMA-IR- Homeostatic Model Assessment for Insulin Resistance, HOMA β-score- Homeostatic Model Assessment for pancreatic β-score.

3.3. Interleukin-6, C - reactive protein and tumor necrosis factor-α concentrations of experimental rats

Interleukin-6, C - reactive protein and tumor necrosis factor-α concentrations in NAD and streptozotocin-induced diabetic rats fed fish oil and Vernonia amygdalina-supplemented biscuits are presented in Table 3.

Table 3. Interleukin-6, C - reactive protein and tumor necrosis factor-α concentrations in NAD and streptozotocin-induced diabetic rats fed fish oil and Vernonia amygdalina-supplemented biscuits.

Groups	IL-6 (pg/mL)	CRP (ng/mL)	TNF-α (mg/mL)
A	2.80 ± 0.10^{a}	0.06 ± 0.01^{a}	25.00 ± 0.12^{a}
B	3.78 ± 0.19^{b}	0.58 ± 0.00^{b}	88.33 ± 0.33^{b}
C	2.94 ± 0.03^{c}	0.04 ± 0.00^{a}	31.00 ± 0.58^{c}
D	2.89 ± 0.11^{d}	0.05 ± 0.01^{a}	25.69 ± 0.16^{a}
E	2.78 ± 0.08^{ad}	0.04 ± 0.00^{a}	25.00 ± 0.28^{a}
F	2.29 ± 0.06^{e}	0.04 ± 0.00^{a}	24.00 ± 0.42^{a}
G	3.43 ± 0.11^{b}	0.58 ± 0.01^{b}	81.00 ± 0.10^{d}

n = 7 ± SEM; mean values with different superscripted alphabets are significantly different ($p < 0.05$), A (Non-diabetic + unsupplemented biscuit), B (diabetic + unsupplemented biscuit), C (Diabetic + 120 mg/kg BW metformin), D (Diabetic + FVSB at 1 g leaf inclusion), E (Diabetic + FVSB at 2 g leaf inclusion), F (Diabetic + FVSB at 3 g leaf inclusion), G (Diabetic + conventional biscuit); FVSB (Fish oil *and Vernonia amygdalina*-supplemented biscuits); IL-6 – Interleukin-6, CRP – C-reactive protein, TNF-α - Tumor necrosis factor-α

Interleukin-6 concentration was significantly elevated ($p < 0.05$) in diabetic untreated rats when compared with the sham control group. There was a significant reduction ($p < 0.05$) in interleukin-6 concentration in rats exposed to FVSB and metformin in comparison to the diabetic untreated group. FVSB treatment (at all leaf inclusion levels) caused a significant reduction ($p < 0.05$) in interleukin concentration than metformin. Rats fed FVSB at 2 g leaf inclusion level showed interleukin concentration comparable ($p > 0.05$) to the sham control group. Interleukin-6 concentration in rats exposed to conventional biscuits did not differ significantly from that of the diabetic untreated rats (Table 3).

The concentration of C-reactive protein was significantly increased ($p < 0.05$) following induction of diabetes. Treatment with FVBS significantly reduced ($p < 0.05$) C-reactive protein concentration when compared with the diabetic untreated group. There was no significant difference ($p > 0.05$) in C-reactive protein concentration of the FVSB-treated group, metformin-treated group and the sham control group. C-reative protein concentration in rats exposed to conventional biscuits did not differ significantly ($p > 0.05$) from that of the diabetic untreated rats (Table 3).

Induction of diabetes significantly increased ($p < 0.05$) the concentration of TNF-α when compared to the sham control group. Exposure to FVSB, metformin and conventional biscuit significantly reduced ($p < 0.05$) the concentration of TNF-α in comparison to the diabetic untreated rats. TNF-α in the FVSB-treated rats was significantly lower ($p < 0.05$) than in the metformin-treated and conventional biscuit-treated groups (Table 3).

4. Discussion

Biscuits are a common snack that can be fortified with medicinal substances to aid in the treatment of certain disorders. Despite the fact that biscuits are widely regarded as harmful due to the high levels of sugar and fat that they contain, there are a number of ways in which they can be made healthier. Fortifying biscuits with nutrition is one method to make them healthier [19], For instance, biscuits that have been fortified with chickpea flour, broad bean flour, and soy protein are those that are considered to be high-protein biscuits. According to the findings of a study conducted in India, biscuits can have thirty percent soy flour added to them in order to increase their nutritional content without affecting their physical features [20]. When researchers added vitamins B12, C, and folic acid to their biscuits, in addition to prebiotic fiber, they were able to transform a conventional biscuit into a functional food. This was accomplished by addition of these ingredients. Participants who consumed these fortified biscuits had reduced blood level of homocysteine and glucose, indicating that the biscuits could aid in lowering risk factors for cardiovascular disease such as heart attack, stroke, and blood clot formation [21]. Similarly, glucomannan extracted from *Amorphophallus konjac* root was incorporated into biscuits, their intake lowered blood glucose levels by 74 % in healthy participants and 63 % in individuals with diabetes [22].

Dietary administration of FVSB elicited a significant hypoglycemic effect in diabetic rats. The glucose-lowering effect of FVSB (at all the leaf inclusion levels tested) recorded in this study was more pronounced than metformin especially after extended exposure duration. Also, the glucose lowering activity of FVSB seemed to be dose and duration dependent, with the 3 g leaf inclusion level performing better than the lower leaf inclusion levels. Although blood glucose concentrations in the treatment groups declined progressively over the 35-day period, euglycemia was not achieved by study termination. The recorded time-dependent decline suggests that a longer intervention may be required to fully restore glycemic control. This glucose-lowering activity of FVSB may be attributed to bioactive compounds contained in V. amygdalina leaf.

Type 2 diabetes is characterised by peripheral insulin resistance and insufficient insulin production. In this study, a derangement of insulin synthesis was recorded following diabetes induction. FVSB counteracted diabetes-occasioned elevation of insulin, with FVSB at 3 g leaf inclusion level restoring insulin concentration to normal level and comparing favourably with metformin. HOMA-IR and HOMA-β score are computational parameters for predicting insulin resistance and pancreatic beta cell functioning respectively. A high HOMA-IR signals the presence of insulin resistance while a low HOMA-β score indicates the beta cell are not working efficiently. The presence of hyperinsulinemia and low HOMA-β score as recorded in this study confirms the successful induction of insulin resistance, with the pancreas working excessively to normalize blood sugar. Although FVSB improved the HOMA-IR and HOMA-β score in the treated animals, normalcy was not achieved before the experiment was terminated. However, FVSB compared favourably with metformin at 3 g leaf inclusion level for HOMA-IR and at all leaf inclusion levels for HOMA-β score. *V. amygdalina* leaf has been reported to contain metabolites such

as vernonoisides, luteolin-7-*O*-β-glucuronoside, and luteolin [23]. These compounds have been shown to stimulate pancreatic β-cell activity via modulation of ATP-sensitive potassium (K_{ATP}) channels and increased Ca^{2+} influx, thereby promoting insulin release [24,25]. Additionally, they enhance peripheral insulin sensitivity through up-regulation of insulin receptor substrate-1 (IRS-1) phosphorylation and activation of the PI3K/Akt signaling cascade, facilitating GLUT4 translocation in skeletal muscle and adipose tissue. Concurrent activation of AMP-activated protein kinase (AMPK) further promotes glucose uptake and suppresses hepatic gluconeogenesis.

Chronic low-grade inflammation is increasingly recognized as a key pathogenic driver in type 2 diabetes mellitus (T2DM) [26,27]. In the present study, the observed anti-inflammatory effects are plausibly attributable to the suppression of pro-inflammatory mediators—specifically interleukin-6 (IL-6), C-reactive protein (CRP), and tumor necrosis factor-alpha (TNF-α)—all of which are implicated in the initiation and progression of T2DM [25,28,29]. Although direct interrogation of inflammatory signaling pathways (e.g., NF-κB activation, IL-6 receptor signaling, or SOCS-3 expression) was beyond the scope of the present work, current evidence supports a mechanistic model involving the attenuation of IL-6 trans-signaling. IL-6 mediates its pro-inflammatory activity primarily via the soluble IL-6 receptor (sIL-6R), which forms a complex with IL-6 to activate the glycoprotein 130 (gp130) receptor subunit on target cells [30–32]. Dysregulated IL-6 trans-signaling has been shown to perpetuate chronic inflammation, exacerbate insulin resistance, and accelerate T2DM progression through induction of suppressor of cytokine signaling 3 (SOCS-3) and subsequent inhibition of insulin receptor substrate-1/2 (IRS-1/2) phosphorylation in insulin-responsive tissues [25]. TNF-α, another key mediator, disrupts insulin signaling via serine phosphorylation of IRS-1, thereby impairing downstream PI3K/Akt activation and glucose uptake [33–36]. CRP, an acute-phase protein and established biomarker of systemic inflammation, is frequently elevated in individuals with T2DM and correlates with disease severity. The significant reductions in IL-6, TNF-α, and CRP observed in the present study therefore suggest that the test supplements may modulate systemic inflammatory responses, potentially alleviating insulin resistance and mitigating the chronic inflammatory milieu characteristic of T2DM. The anti-inflammatory activity observed in the present study is further substantiated by the well-documented bioactive profiles of the supplement constituents. *Vernonia amygdalina* is a rich source of phytochemicals with established anti-inflammatory properties, including luteolin, chlorogenic acid, vernodalin, caffeoyl quinic acid derivatives, and various sesquiterpene lactones [37]. These compounds have been reported to inhibit key pro-inflammatory signaling cascades, notably the NF-κB and mitogen-activated protein kinase (MAPK) pathways, while also modulating cytokine production through down-regulation of TNF-α, IL-6, and other inflammatory mediators. In addition, fish oil, a major source of omega-3 polyunsaturated fatty acids—particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)—has demonstrated potent inflammation-resolving properties. These effects are mediated, in part, by activation of peroxisome proliferator-activated receptor gamma (PPAR-γ), suppression of NF-κB nuclear translocation, and promotion of specialized pro-resolving lipid mediators such as resolvins and protectins [38–40]. The combined presence of these bioactive compounds in the formulated supplement likely produced a synergistic effect, contributing to the attenuation of inflammatory mediators documented in this study.

5. Conclusions

The present study demonstrated that functional biscuits formulated with *Vernonia amygdalina* leaf and fish oil (FVSB) exerted glucose-lowering and anti-inflammatory effects in diabetic rats, potentially mediated through enhanced insulin secretion, improved insulin sensitivity, and suppression of proinflammatory cytokine expression. These findings highlight the potential of FVSB as a functional food candidate for the dietary management of type 2 diabetes.

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Conflict of Interest

Non declared.

Ethical Approval

The study received approval from the Ethical Review Committee (UERC) and the number UERC/LSC 078 was given.

Data Availability

The author confirms that the data supporting the findings in this study are available within the manuscript.

Author Contributions

OS: Conceptualization, supervision, Formal analysis &Writing. **UOA:** Writing, review & editing. **COO:** Review, editing & Investigation. **SOK:** Writing & Formal analysis. **JOO:** Writing, Data curation. **EOO:** Review, editing & Investigation. **AFA:** Writing & Investigation. **HTF:** Writing & editing.

References

[1] Unger RH, Orci L. Paracrinology of islets and the paracrinopathy of diabetes. Proc Natl Acad Sci USA 2010;107(37): 16009–12.

[2] Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. Lancet 2005;365: 1333–46. https://doi.org/10.1016/S0140-6736(05)61032-X.

[3] Weyer C, Bogardus C, Mott DM, Pratley RE. The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. J Clin Investig 1999;104:787–94. https://doi.org/10.1172/JCI7231.

[4] Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006;444:860–7. https://doi.org/10.1038/nature05485.

[5] Johnson AR, Milner JJ, Makowski L. The inflammation highway: metabolism accelerates inflammatory traffic in obesity. Immunol Rev 2012;249:218–38. https://doi.org/10.1111/j.1600-065X.2012.01151.x.

[6] Ceriello A. The emerging challenge in diabetes: the "metabolic memory." Vasc Pharmacol 2012;57:133–8. https://doi.org/10.1016/j.vph.2012.05.005.

[7] Harris MI. Health care and health status and outcomes for patients with type 2 diabetes. Diabetes Care 2000;23:754–8.

[8] Saaddine JB, Engelgau MM, Beckles GL, Gregg EW, Thompson TJ, Narayan KM. A diabetes report card for the United States: quality of care in the 1990s. Ann Intern Med 2002;136:565–74.

[9] Beckles GL, Engelgau MM, Narayan KM, Herman WH, Aubert RE, Williamson DF. Population-based assessment of the level of care among adults with diabetes in the U.S. Diabetes Care 1998;21:1432–8.

[10] Saydah SH, Fradkin J, Cowie CC. Poor control of risk factors for vascular disease among adults with previously diagnosed diabetes. JAMA 2004;291:335–42. https://doi.org/10.1001/jama.291.3.335.

[11] Grant RW, Pirraglia PA, Meigs JB, Singer DE. Trends in the complexity of diabetes care in the United States from 1991 to 2000. Arch Intern Med 2004;164:1134–9. https://doi.org/10.1001/archinte.164.10.1134.

[12] Koro CE, Bowlin SJ, Bourgeois N, Fedder DO. Glycemic control from 1988 to 2000 among U.S. adults diagnosed with type 2 diabetes': a preliminary report. Diabetes Care 2004;27: 17–20.

[13] MacLean CD, Littenberg B, Kennedy AG. Limitations of diabetes pharmacotherapy: results from the Vermont Diabetes Information System study. BMC Fam Pract 2006;7:50. https://doi.org/10.1186/1471-2296-7-50.

[14] Soji-Omoniwa O, Adekeye BT, Abdulazeez UO, Olayinka JO, Oloba CO, Ajediti AF, et al. Effect of *Fish oil and Vernonia amygdalina-supplemented biscuit*s on lipid profile parameters and blood glucose concentration of diabetic rats. Sci World J 2024;19(1):117–22.

[15] Ugbogu EA, Emmanuel O, Dike ED, Agi GO, Ugbogu OC, Ibe C, et al. The phytochemistry, ethnobotanical, and pharmacological potentials of the medicinal plant *Vernonia amygdalina* L. (bitter leaf). Clin Compl Med Pharm 2021;1(1).

[16] Vidotti RM, Pacheco MFVSB, Gonçalves GS. Characterization of the oils present in acid and fermented silages produced from tilapia filleting residue. Rev Bras Zootec 2011;40 (2):240–4.

[17] Whitley PR. Biscuit manufacturer. London, UK: Applied Sciences Publisher Limited; 1970.

[18] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412–9.

[19] Rababah TM, Al-Mahasneh MA, Ereifej KI. Effect of chickpea, broad bean, or isolated soy protein additions on the physicochemical and sensory properties of biscuits. J Food Sci 2006;71:S438–42.

[20] Singh RKP, Sabapathy KK. Opportunities for processing and utilization of soybean to increase nutritional security in North-East India. In: Basu D, Kulirani BF, Ray BD, editors. Agriculture, food security, nutrition and health in North-East India. New Delhi: Mittal Publications; 2006. p. 295–308.

[21] Boobier WJ, Baker J, Davies B. Development of a healthy biscuit: an alternative approach to biscuit manufacture. Nutr J 2006;5:1–7.

[22] Jenkins AL, Jenkins DJA, Wolever TMS, Rogovik AL, Javanovski E, Bozikov V, et al. Comparable postprandial glucose reductions with viscous fiber blend enriched biscuits in healthy subjects and patients with diabetes mellitus: acute randomized controlled clinical trial. Croat Med J 2008; 49:772–82.

[23] Talwar A, Chakraborty N, Zahera M, Anand S, Ahmad I, Siddiqui S, et al. HindawiJournal of chemistry. Article ID 6111603:12 pages. 2024. https://doi.org/10.1155/2024/6111603.

[24] Nkono Ya Nkono BL, Sokeng Dongmo S, Dzeufiet Djomeni PD, Kamtchouing P. Antihyperglycemic and antioxidant properties of *Alstonia boonei* De Wild stem bark aqueous extract in dexamethasone-induced hyperglycemic rats. Int J Diabetes Res 2014;3(3):27–35.

[25] Nkono Ya Nkono BL, Sokeng SD, Njintang Yanou N, Djada AB, Ngoumtsop SS, Koube J, et al. Effects of *Alstonia boonei* aqueous extract in isolated intestinal and diaphragm glucose uptake in vitro. Int J Diabetes Res 2016;5(2):35–40.

[26] Kanmani S, Kwon M, Shin MK, Kim MK. Association of C-reactive protein with risk of developing type 2 diabetes mellitus, and role of obesity and hypertension: a large population-based Korean cohort study. Sci Rep 2019;9(1): 4573. https://doi.org/10.1038/s41598-019-4573.

[27] Lee CC, Adler AI, Sandhu MS, Sharp SJ, Forouhi NG, Erquo S, et al. Association of C-reactive protein with type 2 diabetes: prospective analysis and meta-analysis. Diabetologia 2009;52(6):1040–7. https://doi.org/10.1007/s00125-009-1345-4.

[28] Wang X, Bao W, Liu J, Ouyang Y, Wang D, Rong S, et al. Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis. Diabetes Care 2013;36(1): 166–75. https://doi.org/10.2337/dc12-0926.

[29] Bertoni AG, Burke GL, Owusu JA, Carnethon MR, Vaidya D, Barr RG, et al. Inflammation and the incidence of type 2 diabetes: the Multi-Ethnic Study of Atherosclerosis (MESA). Diabetes Care 2010;33(4):804–10. https://doi.org/10.2337/dc09-1679.

[30] Han TS, Sattar N, Williams K, Gonzalez-Villalpando C, Lean MEJ, Haffner SM. Prospective study of C-reactive protein in relation to the development of diabetes and metabolic syndrome in the Mexico City Diabetes Study. Diabetes Care 2002;25(11):2016–21. https://doi.org/10.2337/diacare.25.11.2016.

[31] Freeman DJ, Norrie J, Caslake MJ, Gaw A, Ford I, Lowe GDO, et al. C-reactive protein is an independent

predictor of risk for the development of diabetes in the West of Scotland Coronary Prevention Study. Diabetes 2002;51(5): 1596–600. https://doi.org/10.2337/diabetes.51.5.1596.

[32] Sproston NR, Ashworth JJ. Role of C-reactive protein at sites of inflammation and infection. Front Immunol 2018;9:754. https://doi.org/10.3389/fimmu.2018.00754.

[33] Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 1999;340(6): 448–54. https://doi.org/10.1056/NEJM199902113400607.

[34] Krogh-Madsen R, Plomgaard P, Møller K, Mittendorfer B, Pedersen BK. Influence of TNF-α and IL-6 infusions on insulin sensitivity and expression of IL-18 in humans. Am J Physiol Endocrinol Metab 2006;291(1):E108–15. https://doi.org/10.1152/ajpendo.00471.2005.

[35] Rask-Madsen C, Domínguez H, Ihlemann N, Hermann T, Kober L, Torp-Pederson C. Tumor necrosis factor-α inhibits insulin's stimulating effect on glucose uptake and endothelium-dependent vasodilation in humans. Circulation 2003;108(15):1815–21. https://doi.org/10.1161/01.CIR.0000091406.72832.11.

[36] Mohallem R, Aryal UK. Regulators of TNFα-mediated insulin resistance elucidated by quantitative proteomics. Sci Rep 2020;10(1):20878. https://doi.org/10.1038/s41598-020-77914-1.

[37] Anh HLT, Vinh LB, Lien LT, Cuong PV, Arai M, Ha TP, et al. In vitro study on α-amylase and α-glucosidase inhibitory activities of a new stigmastane-type steroid saponin from the leaves of *Vernonia amygdalina*. Nat Prod Res 2021;35(5): 873–9. https://doi.org/10.1080/14786419.2020.1715098.

[38] Johnson C, Lin L-Z, Harnly J, Oladeinde FO, Kinyua AM, Michelin R, et al. Identification of the phenolic components of *Vernonia amygdalina* and *Russelia equisetiformis*. J Nat Prod 2011;4:57–64.

[39] Zhou X, Chai L, Wu Q, Wang Y, Li S, Chen J. Anti-diabetic properties of bioactive components from fish and milk. J Funct Foods 2021;85:104669. https://doi.org/10.1016/j.jff.2021.104669.

[40] Sasongko H, Nurrochmad A, Nugroho AE, Rohman A. Fish oil supplementation in diabetic nephropathy prevents inflammatory and oxidative stress target. Food Res 2023;7(2): 307–15.