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Research Article

Red fruit (*Pandanus conoideus* Lam) oil ameliorates streptozotocin-induced diabetic peripheral neuropathy by targeting the oxidative and inflammatory pathways in the spinal cord in a rat model

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Abstract

Diabetic peripheral neuropathy (DPN) is a common comorbid in diabetic patients. Oxidative stress and inflammation are key to DPN's etiology, making them possible therapy targets. Red fruit oil (RFO) may treat DPN due to its antioxidant and anti-inflammatory properties. The RFO effect in the streptozotocin (STZ)-induced DPN model was examined in terms of spinal cord oxidative and inflammatory functions. STZ 55 mg/kg BW intraperitoneally caused DPN. Seven weeks after induction, rats received vehicle, pregabalin, or RFO at 0.3, 0.6, or 1.2 mL/kg BW for three weeks. Post-treatment thermal hyperalgesia and cold allodynia were examined. Measurements of spinal levels of malondialdehyde (MDA), catalase, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), inducible nitric oxide (iNOS), and nuclear factor-kappa beta (NFκB) were used to measure oxidative and inflammatory indices. In addition, spinal histology was examined. All metrics improved after three weeks of RFO treatment. RFO at 0.6 and 1.2 mL/kg BW significantly reduced MDA, TNF-α, IL-6, iNOS, and NFκB while raising catalase levels. These matched thermal and cold stimulus latency improvements. Additionally, STZ-induced spinal cellular damage was reduced. This study suggests that RFO may be an alternate DPN treatment.

Keywords

red fruit oil, diabetic peripheral neuropathy, spinal cord, rat model

Introduction

Diabetic peripheral neuropathy (DPN) is the most common neuropathy worldwide, affecting half of diabetics. This promotes morbidity, lowers quality of life, and raises mortality (Stepanović-Petrović et al. 2017; Ostovar et al. 2020). DPN can impact central and peripheral nerves (Sangoyomi and Oluwole 2021). Previous animal models of DPN showed early-stage hyperalgesia and late-stage hypoalgesia causing hyperglycemia after streptozotocin

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(STZ) induction (Chu et al. 2008; Zahaby 2020; Gateva et al. 2024). In DPN models, the sciatic nerve is most often used to examine nerve damage and chronic pain. This model shows neurotic changes such as capillary nerve tightness, axonal condensation, nerve demyelination, nerve fiber destruction, and nerve damage, which are typical of peripheral nervous system injury (Sasaki et al. 2020). A previous study suggests that increased oxidative stress and activation of peripheral and central nervous system pro-inflammatory cytokines and chemokines induce neurological and neurovascular diseases (Faheem et al. 2022).

Increasing evidence links inflammation to the development and maintenance of neuropathic pain in the peripheral and central nervous systems of the spinal cord. STZ-induced neuropathic pain is affected by spinal cord microglia and astrocyte activation (Jia et al. 2020). Some studies suggest that peripheral inflammation can increase nuclear factor-kappa beta (NF-κB) in the spinal cord (Elsayed et al. 2023), leading to the production of several pro-inflammatory chemokines and cytokines, such as tumor necrosis factor-alpha (TNF-α) (Alomar et al. 2021) and interleukin-6 (IL-6) (Ni et al. 2023). Additionally, NF-κB is linked to inducible nitric oxide (iNOS) production and inflammatory and neuropathic pain development (Naveed et al. 2021). Increasing proinflammatory cytokines, neuroinflammation, and oxidative stress can increase pain sensitivity. These alterations may cause central sensitization and neuropathic pain in diabetes models (Bishnoi et al. 2011). Oxidative stress and inflammation have a major impact on the pathogenesis of diabetic neuropathy, making natural products an interesting study in alternative therapy. Red fruit (*Pandanus conoideus* Lam) is a plant native to the Papua Island of Indonesia. The plant's oil (red fruit oil, RFO) is a natural product that is widely used in the food and medical fields (Sarungallo et al. 2016). RFO is reported to have antidiabetic (Khairani et al. 2023), anti-inflammatory (Rhee et al. 2020), and antioxidant (Al failah et al. 2024) properties. Red fruit extract can also reduce serum TNF-α levels and increase serum IL-10 concentrations (Felle et al. 2013). In a previous study, increased levels of malondialdehyde (MDA) could also be completely normalized by RFO treatment (Sugiritama et al. 2016). Early research results also show that red fruit oil increases GPx (Sinaga and Susanti 2018). In addition, the ethanol extract of red fruit is also known to reduce TNF-α levels (Tafor et al. 2015). Despite this cumulating evidence, however, the effect of RFO on DPN, let alone the mechanisms of action, is yet to be proven.

In this study, we developed an STZ-induced DPN rat model followed by an investigation of the effect of an RFO intervention. The success of induction in diabetes assessment was observed by increased blood glucose levels, weight loss, and pancreatic damage, while the DPN model was confirmed by thermal hyperalgesia and cold allodynia. The involvement of oxidative and inflammatory pathways was assessed by measuring spinal levels of MDA, catalase, and relevant cytokines. Spinal histologic consequences were also observed.

Materials and methods

Drugs and chemicals

Red Fruit Oil used in this study was commercial herbal produced by PT. Hujan Bahagia, West papua, Indonesia. Streptozotocin (STZ) was purchased from Bioworld. Pregabalin (PGB) was purchased from PT. Pharos Indonesia. Sodium carboxymethyl cellulose (CMC-Na), phosphate-buffered saline (PBS), potassium dichromate ($K_2Cr_2O_7$), glacial acetate, tetra ethoxy propane, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethanol (70%, 80%, and 90%), xylol, hematoxylin, and eosin (HE), and hydrogen peroxide (H_2O_2) were purchased from Merck. Bovine serum albumin (BSA) was purchased from Himedia. TNF-α and IL-6 ELISA kits were purchased from Elebsains, Shanghai. NFκB and iNOS ELISA kits were purchased from BT Lab, China.

Test animals and induction

To calculate the number of samples per treatment group, the Federer formula was used: (n-1) (t-1) ≥ 15 (where t is the treatment group). The minimum sample size is four because the treatment group is six. A total of 24 male Wistar rats, having an initial weight of 250–300 g aged 12–15 weeks, were used. The rats were maintained at room temperature of 23 ± 2 °C and relative humidity of 55 ± 10% with a 12 h/12 h light/dark cycle. This experiment was approved by the Bandung Institute of Technology Animal Ethics Committee (approval ID: KEP/I/2024/II/H050224IT/MDNS).

After fasting for one day, rats were injected intraperitoneally with STZ 55 mg/kg in 0.1 mol/L citrate buffer (pH 4.5) to induce diabetes. Diabetes was defined as fasting blood glucose (FBG) levels above 200 mg/dL in rats used in follow-up tests. The rats were separated into five groups six weeks following diabetes confirmation, as shown in Fig. 1. Treatment was carried out after six weeks because symptoms of diabetic neuropathy such as mechanical, thermal, and chemical hyperalgesia in male Wistar rats generally began to be observed after six weeks of induction (Sandireddy et al. 2016; Hristov et al. 2024). The preparation was administered for 3 weeks via the oral route. After verification of the neuropathic model with cold hyperalgesia and allodynia testing. The dosage of RFO given in this study follows from Sinaga et al. (2020).

Measurement of body weight and fasting blood glucose levels

Body weight is measured by analytical balance. Blood glucose levels were measured by briefly puncturing the tail vein. Use a portable glucometer (Autogluco.DR) (Borsha et al. 2020).

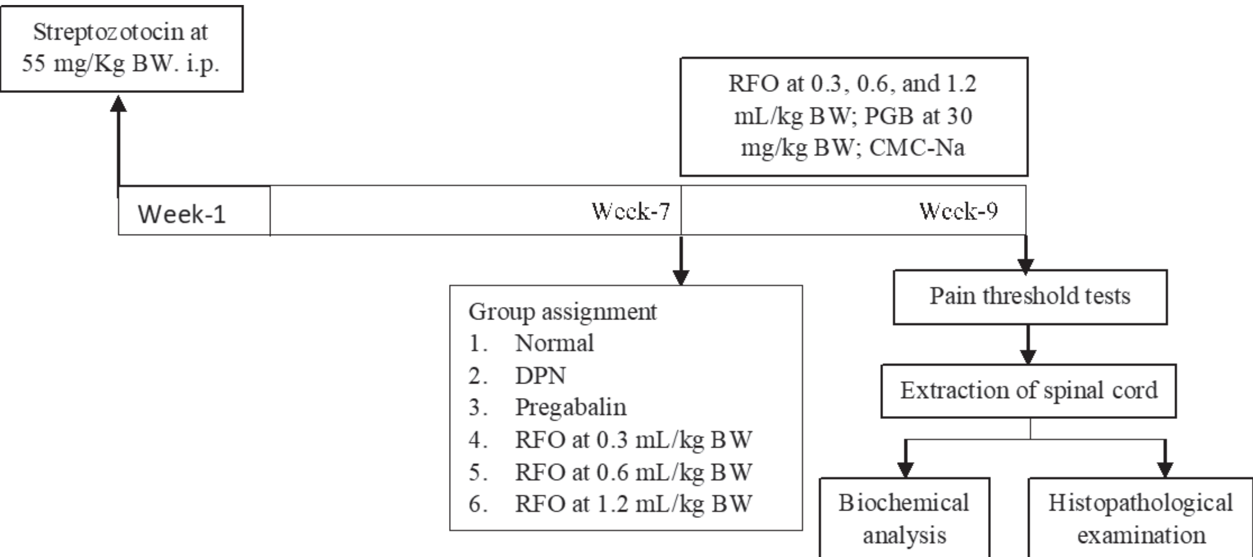

Figure 1. Experimental design. The animals were adapted for 7 days before the trial. After fasting for one day, rats were injected intraperitoneally with streptozotocin at 55 mg/kg BW in 0.1 mol/L citrate buffer (pH 4.5) to induce hyperglycemia. RFO was given daily at. 0.3 mL, 0.6 mL, and 1.2 mL/kg BW for 3 weeks. Pregabalin (30 mg/kg BW) was given as a reference drug. CMC-Na was given to the normal control and DPN groups. At the end of testing, all groups were sacrificed for biochemical analysis and histopathological examination.

Effect on behavioral changes

Cold allodynia

Cold allodynia tests involved tail immersion. A 5 cm portion of the tail was submerged in 10 ± 2°C. The time taken to remove the tail from cold water was noted. For tissue protection, a 15-second timeout was implemented. For each rat, three measurements were taken to establish the average time. Five minutes of separate repetitions (Al-Massri et al. 2018)

Thermal hyperalgesia

The rat's tail was immersed in warm water (45 ± 2°C) after a period of acclimatization. A tissue injury was avoided with a 12-second cutoff. Each rat was measured three times with a 5-minute interval. Next, the average time was calculated (Bachewal et al. 2018).

Oxidative pathways involvement

Homogenate preparation

This study used lumbar L4-6 spinal cord tissue. A histological study was performed on the 0.5 cm spinal cord, while other biological contents were examined on the remaining nerves. A total of 10% homogenate was crushed in 50 mM phosphate buffer (pH 4.5) (1:9) after tissue washing with cold saline. Centrifuge at 4000 rpm at 4 °C for 10 minutes (Salem et al. 2020).

Malondialdehyde content analysis

Measurement of MDA levels was carried out following a method by (Papastergiadis et al. 2012). A total of 0.5 mL of sample was added with 0.5 mL of 20% TCA and 1 mL of 0.67% TBA, then shaken until homogeneous. This combination is heated at 95–100 °C for 10 minutes. After cooling, this mixture was centrifuged at 4000 rpm for 10 minutes. The absorbance of the supernatant was measured at a wavelength (λ) of 532 nm. To prepare a standard curve, a standard solution of tetraethoxypropane (dilution 1/80,000 times) was used. From this solution, 10, 20, 40, 80, and 160 µL were taken and put into a test tube. Next, distilled water was added up to 1 mL and shaken homogeneously. Then 0.5 mL of 20% TCA and 1 mL of 0.67% TBA solution were added to each tube and shaken until homogeneous. The blank solution was made by mixing 1 mL of distilled water, 0.5 mL of 20% TCA solution, and 1 mL of 0.67% TBA solution, then shaking until homogeneous. The blank standard solution was made in duplicate. All tubes were placed in a 95–100 °C water bath for 10 minutes, then cooled in running water. Absorption was measured at a wavelength of 532 nm. From the measurement data, a calibration curve was created by connecting the absorption value as the coordinate (Y) and the standard solution concentration (nmol/mL) as the abscissa (X). The concentration of the MDA sample is expressed (in nmol/mL).

Measurement of catalase activity

The measurement of catalase activity was carried out following the Sinha method with modifications by Hadwan and Abed (2016). Samples 100 µl were reacted with 1000 µl H_2O_2 (65 mM) in phosphate buffer (50 mM, pH 7.4) containing 0.1% w/v BSA, vortexed, and incubated for 37°C. The next sample was added dichromate in acetic acid glacial. After that, the tube was kept at 100 °C for

10 minutes. The activity was stopped by cooling the sample; the sample was centrifuged to remove the precipitated protein (2500 g for 5 minutes), and the absorbance change was recorded at 570 nm against the blank reagent. Catalase activity sample concentration is represented in units per mL (U/mL).

Effect on inflammation biomarkers

Inflammatory cytokines

The TNF-α and IL-6 levels in rat spinal cord tissue were tested using a protocol ELISA kit (Elebsains, China). Sample homogenate (100 μL) was added to the ELISA well kit and incubated for 90 minutes. After discarding the sample, 100 μL of biotinyl AB detection was applied and incubated for 60 minutes. The sample was discarded, washed with 350 μL wash buffer 5×, and dried. The sample was added with 100 μL of HRP conjugate and incubated for 30 minutes. The sample was discarded, washed with 350 μL wash buffer 5×, and dried. After adding 90 μL of substrate, the sample was incubated for 15 minutes. The sample was added with 50 μL of stop solution and incubated for 15 minutes. An ELISA reader (Tecan) read the sample at 417 nm. Sample concentrations of TNF-α and IL-6 are quantified (in pg/mL).

Other inflammatory mediator

The other inflammatory mediators, NFκB and iNOS, were evaluated using a separate ELISA kit (BT Lab, China). Sampel homogenate (40 μL) was added to the ELISA well kit and incubated for 90 minutes. The sample was discarded, and 10 μL of antibody was added. The sample was added with 50 μL of HRP conjugate and incubated for 60 minutes at 37°C. The sample was discarded, washed with 5× wash buffer in 350 μL, and dried. The sample was added with 50 μL of substrate A and 50 μL of substrate B and incubated for 10 minutes at 37°C. The sample was added with 50 μL of stop solution and incubated for 10 minutes. An Elisa reader (Tecan) read the sample at 417 nm. Sample concentrations of NFκB and iNOS concentrations are expressed (in ng/mL).

Histopathological changes

DPN rats were sacrificed by CO_2 gas. Spinal cord tissue was preserved in 10% buffered formalin. Dehydrating the tissue with 70%–100% graded ethanol followed fixation. Dehydrated tissue was cleaned with xylol. Paraffin wax melted at 60 °C was used to implant the tissue. To remove wrinkles, the tissue was immersed in 40 °C water. The tissue was dried in an incubator for two nights on a glass slide. Next, 100% xylene deparaffinized the tissue slides, ethanol gradients of 70–95% concentration rehydrated them, and distilled water cleaned them. After 3–8 minutes of hematoxylin incubation, slides were washed with running water for 20 minutes. The slide preparations were cleaned again with 70–90% ethanol. A 30-second soak in eosin solution was followed by two rapid dips in 94% alcohol. Slides were dried for 15 minutes and fixed with xylol for 1–2 minutes. Following slide preparation, 1–2 drops of Entellan mounting medium were added and covered with a cover glass. The slides were dried for 15 minutes and inspected under a microscope, with images acquired at ×4 and ×10 magnification (Khan et al. 2021). Quantification of histopathology results was carried out using blood vessel dilation parameters calculated using the ImageJ program; each group used four replications with different fields of view.

Data analysis

Behavioral and neurochemical data were presented as mean ± SD. The data was analyzed using Statview software to determine normality (Shapiro-Wilk) and between-group differences (one-way ANOVA with Bonferroni-Dunn post hoc). P-values under 0.05 were set for significance.

Results

Glucose levels and body weight observations

The confirmation of STZ induction was assessed by blood glucose level increase and body weight reduction. Profiles of glucose levels and body weight of the test animals and the effects of RFO are presented in Fig. 2.

Following RFO treatment, a one-way ANOVA revealed a significant effect of treatment ($F(5,18)=45.18$, $P < 0.05$). The DPN group (538.00 ± 66.57 mg/dL, $P < 0.05$) showed significantly higher glucose levels than the normal group (90.00 ± 3.87 mg/dL). Pregabalin (479.5 ± 24.89 mg/dL, $P > 0.05$) had no significant effect on glucose level compared to the DPN group. RFO dose 0.3 ml/kg BW (426.50 ± 38.41 mg/dL, $P < 0.05$), RFO dose 0.6 (358.3 ± 79.02 mg/dL, $P < 0.05$), and RFO dose 1.2 mL/kg BW (331.3 ± 17.33 mg/dL, $P < 0.05$) significantly decreased glucose levels but had not returned to the normal level.

Upon RFO treatment, a one-way ANOVA revealed a significant effect of treatment ($F(5,18)= 38.57$, $P < 0.05$). The DPN rats (213.80 ± 5.12 grams, $P < 0.05$) had significantly lower average body weight compared to the normal group (324.5 ± 18.19 grams). There was no significant weight difference found between DPN and pregabalin (218.3 ± 15.17 grams, $P > 0.05$). Administration of RFO at 0.3 mL/kg BW (242.80 ± 9.639 grams, $P < 0.05$), RFO dose 0.6 mL/kg BW (242.80 ± 9.639 grams, $P < 0.05$), and RFO dose 1.2 mL/kg BW (250.30 ± 9.39 grams, $P < 0.05$) significantly increased body weight compared with the DPN group, but had not returned to the normal level.

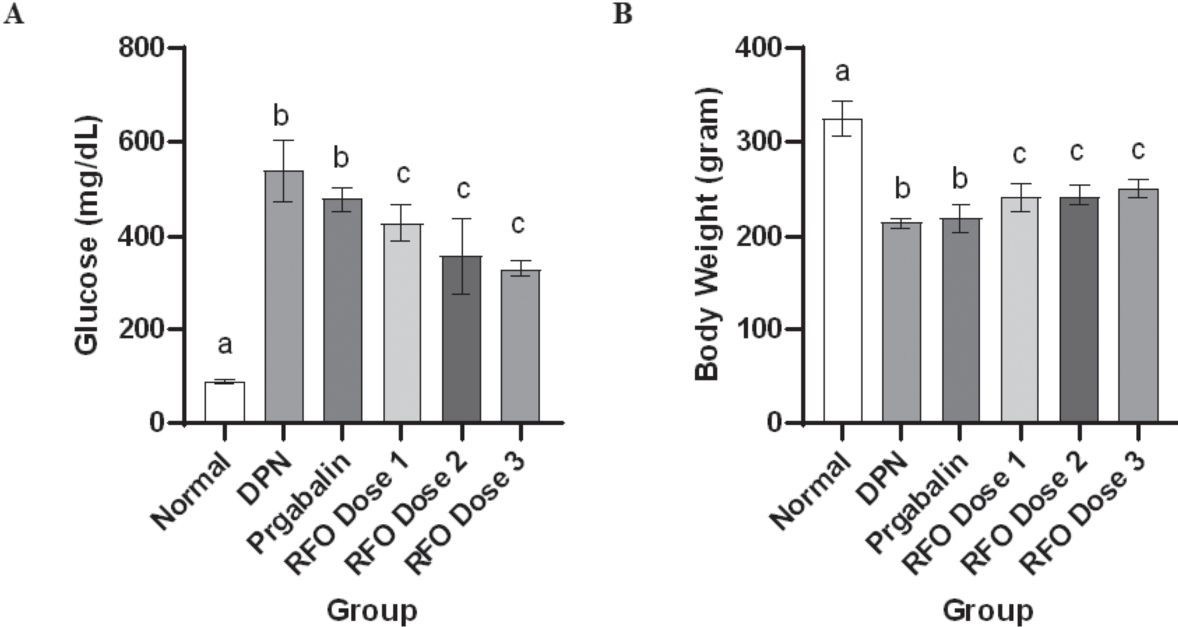

Figure 2. RFO therapy in STZ-induced diabetic rats affects blood glucose concentration **(A)** and body weight **(B).** Treatments were initiated six weeks post-induction and lasted for three weeks. Data represents the average ± SD of four replications. One-way ANOVA followed Bonferroni-Dunn *post hoc*. Doses: Pregabalin = 30 mg/kg BW; Red fruit oil (RFO): Doses 1, 2, and 3 = 0.3, 0.6, and 1.2 mL/kg, respectively. All values with different letters are significantly different ($P < 0.05$).

Effect on stimuli threshold

Cold allodynia and thermal hyperalgesia

Diabetic neuropathy causes decreased sensory responsiveness to hot and cold temperatures, as presented in Fig. 3.

Following RFO treatment, a one-way ANOVA revealed a significant effect of treatment ($F(5,18 = 45.76$, $P < 0.05$). Latency time in the cold allodynia test was significantly lower in the DPN group (4.41 ± 0.34 seconds, $P < 0.05$) compared to the normal group (14.03 ± 1.78 seconds). Pregabalin significantly increased the latency (11.01 ± 1.35 seconds, $P < 0.05$) compared with the DPN but did not return to normal condition. RFO 0.3 mL/kg BW (5.02 ± 0.74 seconds, $P > 0.05$) was insignificant compared with the DPN group. Administration RFO 0.6 mL/kg BW (6.29 ± 1.24 seconds, $P < 0.05$) and 1.2 mL/kg BW (6.61 ± 0.54 seconds, $P < 0.05$) increased the latency time significantly compared with the DPN group.

Upon RFO treatment, a one-way ANOVA revealed a significant effect of treatment ($F(5,18 = 78.51$, $P < 0.05$). Latency time in the thermal hyperalgesia test was significantly lower in the DPN group (3.99 ± 0.60 seconds, $P < 0.05$) compared to the normal group (11.83 ± 0.90 seconds). Pregabalin significantly increased the latency (9.30 ± 0.68 seconds, $P < 0.05$) compared with the DPN but did not return to normal condition. RFO 0.3 mL/kg BW (4.67 ± 0.488 seconds, $P > 0.05$) was insignificant compared to the DPN group. Administration RFO 0.6 mL/kg BW (6.50 ± 0.58, $P < 0.05$); and RFO 1.2 mL/kg BW (6.49 ± 0.65 seconds, $P < 0.05$) show an increase in the latency time compared with the DPN group.

Oxidative pathway involvement

Abnormal oxidative stress in diabetic neuropathy can be characterized by changes in malondialdehyde (MDA) level and catalase activity, as shown in Fig. 4.

Upon RFO treatment, a one-way ANOVA revealed a significant effect of treatment ($F(5,18)=62.16$, $P < 0.05$). MDA levels in the DPN group (205.20 ± 11.65 nmol/mL, $P < 0.05$) were significantly increased when compared with the normal group (129 ± 4.33 nmol/mL). Pregabalin (144.80 ± 4.70 nmol/mL, $P < 0.05$) significant decrease compared with the DPN group. RFO at 0.3 (174.30 ± 5.03, nmol/mL, $P < 0.05$) and RFO at 0.6 ml/kg (144 ± 12.39 nmol/mL, $P < 0.05$) significantly decreased compared to the DPN group, but the level had not returned to a normal level. The group treated with RFO 1.2 ml/kg BW (121.90 ± 4.21 nmol/mL, $P < 0.05$) had significantly lower MDA levels compared with the DPN group, and the level had returned to a normal level.

Following RFO treatment, a one-way ANOVA revealed a significant effect of treatment ($F(5,18)= 5.572$, $P < 0.05$). Catalase activity was significantly decreased in the DPN group (1.79 ± 0.24 U/mL, $P < 0.05$) when compared with the normal group (2.49 ± 0.13 U/mL). Pregabalin (2.07 ± 0.100 U/mL, $P > 0.05$) and RFO at 0.3 ml/kg BW (2.10 ± 0.12 U/mL) increased levels of catalase, but not yet significant compared to the DPN group. RFO dose 0.6 mL/kg BW (2.37 ± 0.30 U/mL, $P < 0.05$) and RFO dose 1.2 ml/kg BW (2.34 ± 0.28 U/mL, $P < 0.05$) a significant increase in catalase activity compared to DPN group.

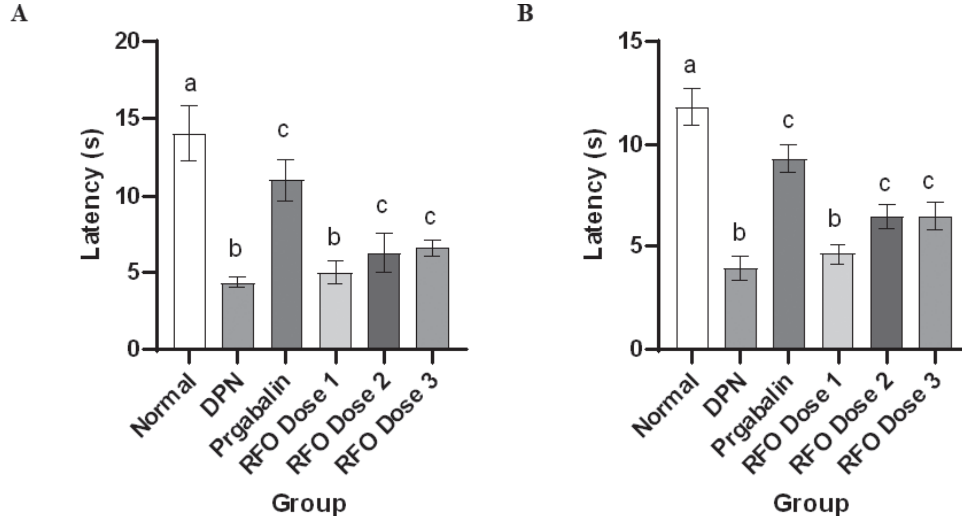

Figure 3. RFO therapy impacts cold allodynia (**A**) and thermal hyperalgesia (**B**) in STZ-induced diabetic rats. Treatments were initiated six weeks post-induction and lasted for three weeks. Data represents the average ± SD of four replications. One-way ANOVA followed Bonferroni-Dunn *post hoc*. Doses: Pregabalin = 30 mg/kg BW; Red fruit oil (RFO): Doses 1, 2, and 3 = 0.3, 0.6, and 1.2 mL/kg, respectively. All values with different letters are significantly different (P < 0.05).

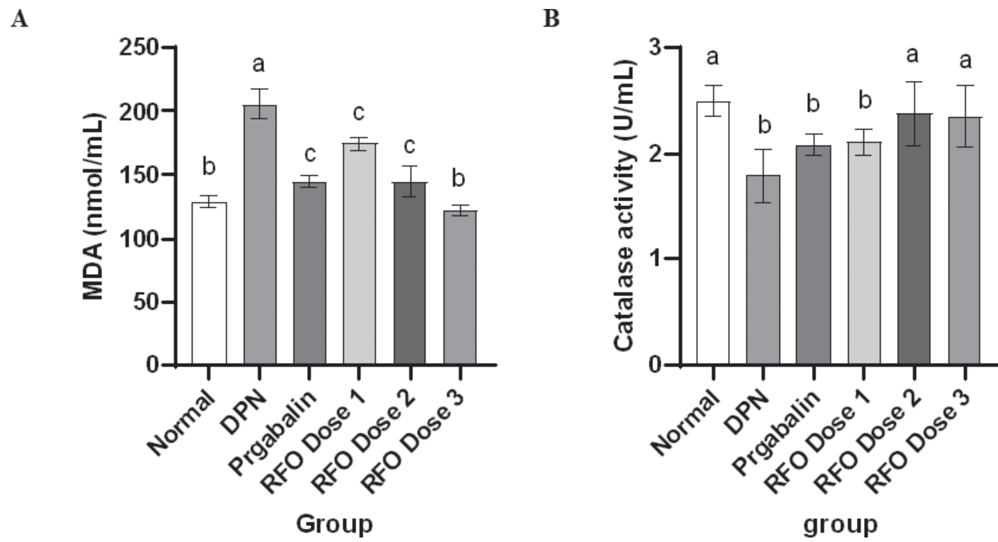

Figure 4. RFO therapy impacts in MDA level **(A)** and catalase activity **(B)**. Treatments were initiated six weeks post-induction and lasted for three weeks. Data represents the average ± SD of four replications. One-way ANOVA followed Bonferroni-Dunn *post hoc*. Doses: Pregabalin = 30 mg/kg BW; Red fruit oil (RFO): Doses 1, 2, and 3 = 0.3, 0.6, and 1.2 mL/kg, respectively. All values with different letters are significantly different (P < 0.05).

Inflammatory pathway involvement

Measurement of inflammatory cytokines

The consequences of RFO treatment on the inflammatory cytokines TNF-α and IL-6 are presented in Fig. 5.

Following RFO treatment, a one-way ANOVA revealed a significant effect of treatment (F(5,18)= 9.918, P < 0.05). The DPN group (59.04 ± 16.36 pg/mL, P < 0.05) showed significantly higher TNF α than compared to the normal group (23.36 ± 16.36 pg/mL). Pregabalin (25.55 ± 4.24 pg/mL, P < 0.05) significantly decreased compared with the DPN group. Administration of doses 0.3 ml/kg BW (36.58 ± 11.27 pg/mL, P < 0.05) had no significant effect compared to the DPN group. RFO doses of 0.6 ml/kg BW (29.36 ± 4.256 pg/mL, P < 0.05) and RFO doses of 1.2 ml/kg BW (24.54 ± 3.223 pg/mL, P < 0.05) significantly decrease TNF α compared with the DPN group.

Upon RFO treatment, a one-way ANOVA revealed a significant effect of treatment (F(5,18)= 20.89, P < 0.05). DPN group (212.2 ± 16.31 pg/mL, P < 0.05) showed significantly higher IL-6 than normal (152.8 ± 8.159 pg/mL). Pregabalin (150 ± 5.66 pg/mL, P < 0.05) significantly reduced concentration compared to the DPN group. RFO 0.3 ml/kg BW (200.8 ± 16.63 pg/mL, P > 0.05) was not significantly different compared with the DPN group. RFO doses of 0.6 mL/kg BW (176.5 ± 6.439 pg/mL, P < 0.05) significantly decreased compared to the DPN group, but the level had not returned to a normal level. RFO doses of 1.2 ml/kg (168.2 ± 6.994 pg/mL, P < 0.05) were shown to significantly reduce IL-6 compared to the DPN group.

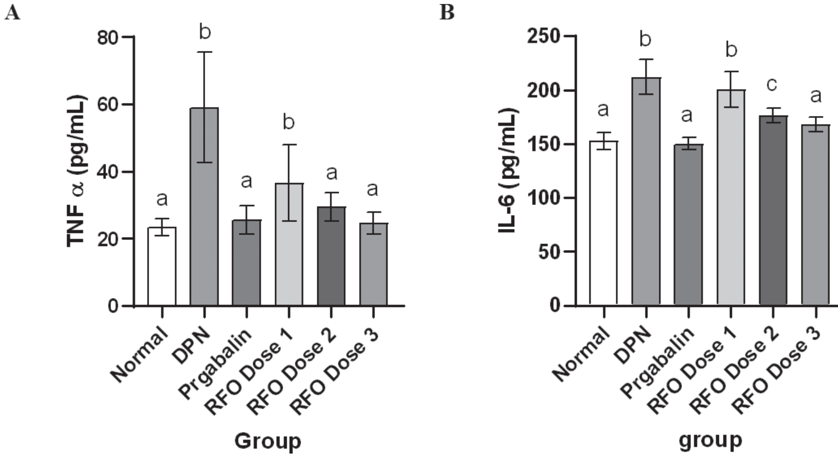

Figure 5. Effects of RFO therapy on TNF-α (**A**) and IL-6 (**B**) levels. Treatments were initiated six weeks post-induction and lasted for three weeks. Data represents the average ± SD of four replications. One-way ANOVA followed Bonferroni-Dunn *post hoc*. Doses: Pregabalin = 30 mg/kg BW; Red fruit oil (RFO): Doses 1, 2, and 3 = 0.3, 0.6, and 1.2 mL/kg, respectively. All values with different letters are significantly different (P < 0.05).

Other inflammatory mediator

The consequences of RFO treatment on the inflammatory mediators iNOS and NFkB are presented in Fig. 6.

After RFO treatment, a one-way ANOVA revealed a significant effect of treatment (F(5,18)= 4.460, P < 0.05). The DPN group (58.00 ± 3.89 ng/mL, P < 0.05) showed significantly higher iNOS levels compared with the normal group (47.02 ± 6.38 pg/mL). Pregabalin (49.73 ± 3.02 ng/mL, P > 0.05) and RFO dose 0.3 mL/kg BW (51.43 ± 4.35 ng/mL, P > 0.05), RFO dose 0.6 mL/kg BW (47.77 ± 4.42 ng/mL, P < 0.05), and RFO dose 1.2 mL/kg BW (45.90 ± 0.829 ng/mL, P < 0.05) significantly reduced iNOS compared to the DPN group.

Following RFO treatment, a one-way ANOVA revealed a significant effect of treatment (F(5,18)=4.173, P < 0.05). DPN group (4.788 ± 0.59 ng/mL, P < 0.05) had significantly higher NFkB levels than normal (3.28 ± 0.40 ng/mL). Administration of pregabalin (3.62 ± 0.33 ng/mL, P < 0.05) significantly lowered concentration compared to the DPN group. RFO 0.3 mL/kg BW (4.45 ± 0.92 ng/mL), 0.6 mL/kg BW (4.20 ± 0.30 ng/mL), and RFO doses 1.2 mL/kg BW (4.32 ± 0.46 ng/mL, P < 0.05) decrease NFkB but not significantly compared to the DPN group.

Histopathological observation of the spinal cord

Photomicrograph images of the spinal cord are presented in Fig. 7. The spinal cord from normal controls showed predominant normal nerve cells (black arrows) with scattered glial cells (white arrows), while the spinal cord of the DPN rat indicated some degree of cellular morphological changes. Thus, dilated capillaries (yellow arrow) and gliosis (red arrow) were observed. In the groups given RFO, deviation from normal spinal cord, such as the presence of vacuoles (green arrow), was observed. However, improvements were also seen in the pregabalin-treated group.

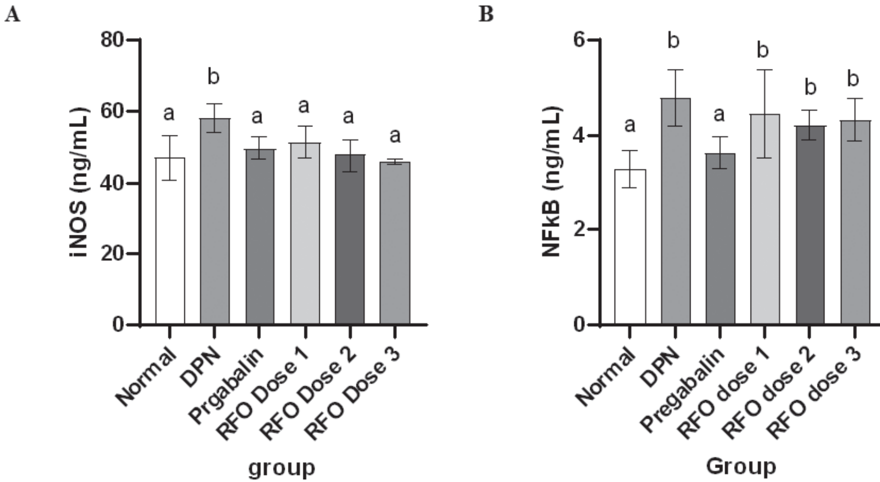

Figure 6. Effects of RFO therapy on iNOS (**A**) and NFkB (**B**) levels. Treatments were initiated six weeks post-induction and lasted for three weeks. Data represents the average ± SD of four rats. One-way ANOVA followed Bonferroni-Dunn *post hoc*. Doses: Pregabalin = 30 mg/kg BW; Red fruit oil (RFO): Doses 1, 2, and 3 = 0.3, 0.6, and 1.2 mL/kg, respectively. All values with different letters are significantly different (P < 0.05).

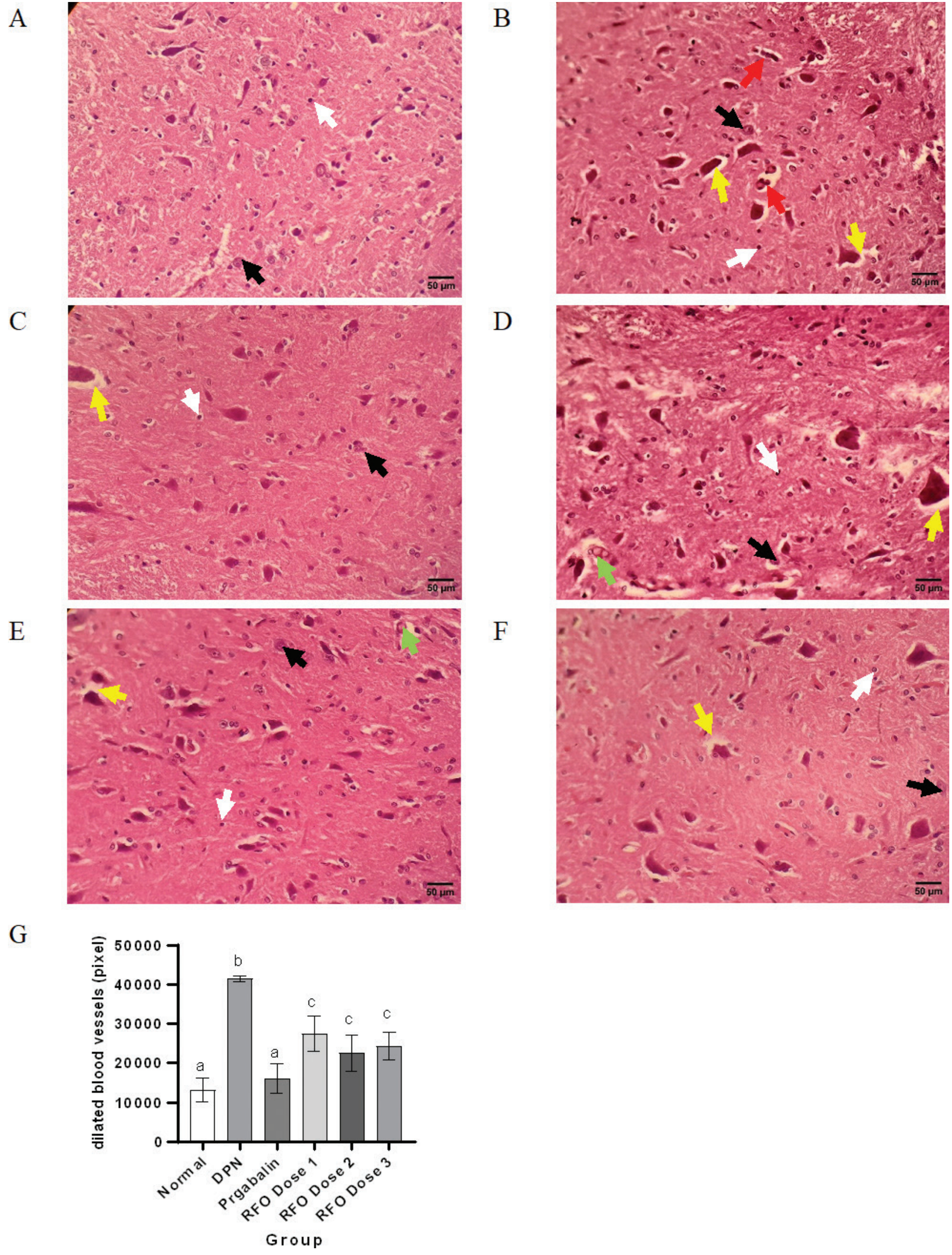

Figure 7. Histopathological presentation of the spinal cord. **A**. Normal; **B**. DPN; **C**. PGB; **D**. RFO dose 0.3 mL/kg; **E.** RFO dose 0.6 mL/kg, and **F**. RFO dose 1.2 mL/kg; **G**. Quantitative assessment of histopathological observation. Arrows: Black = normal nerves, White = glial cells, Yellow = dilated capillaries, Green = vacuoles in neutrophils, Red = gliosis. Magnification: 400×.

Discussion

Neuropathy is the most prevalent diabetes consequence. The streptozotocin-induced diabetes rat model is most typically used to study diabetic neuropathy pain and natural medicines. Markers of diabetic neuropathy in diabetic mouse models were determined by observing behaviors such as thermal hyperalgesia and cold allodynia. This decrease in latency is consistent with previous research findings (Ghatak and Panchal 2012; Adhikari et al. 2015; Stalin et al. 2016; Kiasalari et al. 2017; Singh et al. 2020; Pușcașu et al. 2023).

Histopathological findings reinforce the behavioral test results, which showed changes such as spinal cord capillary dilatation and gliosis, indicating glial activation. Recent research suggests that oxidative stress in STZ-induced diabetic rats causes spinal cord glial activation, leading to peripheral nerve fiber damage (C, A∂, and Aβ) and possible hypersensitivity reactions. Signaling defects between neurons and glia play an important role in the context of maladaptive plastic pain. After activation, glial cells express microglial markers and release various inflammatory mediators, chemokines, and cytokines (Petrou et al. 2013; Zhao et al. 2017). Active glia activates MAPK, NF-κB, and STAT3 to cause neuronal death. Glutamate, ion homeostasis, and energy metabolism activate astrocytes. Activation of microglia results in elevated PICs (IL-6, TNF-α, IL-1β, and COX-2), which directly cause DPN (Spagnuolo et al. 2018; Zhong et al. 2018; Palpagama et al. 2019; Abo Salem et al. 2020).

Diabetic neuropathy also causes spinal cord oxidative stress. Oxidative stress and free radical-induced lipid breakdown are indicated by MDA levels. The results of this study showed an increase in MDA in the spinal cord DPN group, in line with previous studies (Zhao et al. 2014; Patel and Lau-Cam 2017; Villarreal et al. 2020; n.d.). In the case of neuropathic diabetes, a decrease in spinal catalase levels was observed in this study, which is consistent with previous studies (X. Zhao et al. 2015; Patel and Lau-Cam 2017; Ismail et al. 2018).

Previous investigations found elevated IL-6 in the spinal cord of diabetic neuropathy (Liu et al. 2019; Zhou et al. 2019; S. Li et al. 2021). IL-6 expression in the spinal cord can produce bone marrow hyperexcitability in the hind legs due to glial cell activation, inflammatory cytokines, and a considerable rise in p-NR2B expression in the spinal dorsal horn. Kummer et al. (2021) discovered that kinase activation, neuronal excitability regulation, ion channel expression, and signal transducers linked IL-6 levels in neuropathic and inflammatory pain to thermal and mechanical hyperalgesia. This showed a connection between neuropathic pain and inflammation (allodynia), which will become GP130.

The pro-inflammatory cytokine TNF-α contributes to the development of diabetic neuropathy. Excessive TNF-α release causes glutamate buildup, leading to pain behavior, excitotoxicity, and neuronal damage (Clark and Vissel 2016; Kolahdouz et al. 2021). The DPN group had higher levels of spinal TNF-α, consistent with earlier research (Y. Li et al. 2013; Lu et al. 2017; He et al. 2020; T. Li et al. 2021; Shamsipour and Shariati 2023). The necrotic TNF-α pathway, or TNF-α in neuropathic pain, may contribute to peripheral and central sensitization. TNF-α-induced necrosis in the DRG, spinal cord, and supraspinal areas may be a key element in neuropathic pain's neuroimmune response. TNF-α promotes NFκB, leading to NOS (NO synthase) activation and NO synthesis, a pain neurotransmitter. According to Y. Li et al. (2013), several neuropathological diseases associated with hyperalgesia have increased concentrations of TNF-α in the spinal cord and dorsal horn (Choi et al. 2010; Spicarova et al. 2011). TNF-α, a pro-inflammatory cytokine, is believed to initiate signaling cascades (Zhang et al. 2011).

Increased NO levels via iNOS contribute to neuroinflammatory and neurodegenerative disorders in humans. NO is a key spinal cord neurotransmitter. In response to peripheral stimuli, adenosine triphosphate, substance P, and excitatory amino acids damage cells and activate glial cells by attaching to their receptors. Active microglia produce lots of NO and transmit iNOS. Astrocytes and pain-transmitting neurons are activated by it. After induction, iNOS generates NO until degradation (Garry et al. 2015). In line with this study, others have shown that iNOS (expressed centrally and peripherally) contributes to inflammatory and neuropathic pain (H. Li et al. 2020). Activation of NFκB increases transcription of several inflammatory cytokines and stimulates NF-κB, a positive feedback loop that amplifies inflammatory signals and continuously initiates and maintains amplified pain signals. An increase in NFκB in the spinal cord DPN group was also shown by (Alomar et al. 2021; Ni et al. 2023).

PGB decreases spinal cord injury-induced astrocyte proliferation and phosphorylated caspase-3 and p38 MAPK (Ha et al. 2008). In rats, PGB had histopathologically detectable anti-edema, anti-inflammatory, and neuroprotective effects when administered to limit acute brain damage (Calikoglu et al. 2015; Demir et al. 2021). Research suggests that PGB lowers TNFα, NFκB, and iNOS levels in the spinal cord (Faheem et al. 2022). Diabetes rats' PGB tests indicated a considerable drop in MDA but no increase in catalase activity. This may be because PGB reduces pain and inflammation using GABA but does not boost antioxidant activity.

In this study, RFO improves DPN mainly through anti-inflammatory and antioxidant properties (Al failah et al. 2024). Red fruit oil contains components such as omega 3, omega 9, and omega 6, as well as β-carotene and tocopherol. All of these compounds act as antioxidants to prevent lipid oxidation or MDA in cellular membranes. β-carotene can enhance blood sugar regulation and insulin responsiveness by collecting singlet oxygen ($1O_2$) and regulating free radical generation. Beta-carotene boosts antioxidants and reduces free radicals. Beta-carotene prevents hyperglycemia complications and cell damage by inhibiting lipoprotein oxidation (Mohammed et al. 2006). Meanwhile, alpha-tocopherol also inhibits protein kinase C (PKC) to prevent superoxide radicals from forming in monocytes and neutrophils. Prevention of PKC autophosphorylation or dephosphorylation by alpha-tocopherol-activated protein phosphatase inhibits PKC (Armiyanti et al. 2007). Sinaga et al. (2020) Tocopherol in red fruit oil can easily donate hydrogen atoms to free radicals' hydroxyl group, making them non-reactive. Tocopherol becomes a radical by donating hydrogen, but it is more stable because the oxygen atom's unpaired electrons are delocalized into the aromatic ring structure. RFO inhibits MAPK and NF-κB signaling, leading to reduced ROS (Rhee et al. 2020). The increased catalase activity in RFO treatment was studied by Umar et al. (2021) through a molecular docking study. The test results showed that antioxidant compounds in red fruit, such as 3-glucoside, bind more strongly to catalase CAT, GR,

GPx, SOD, LOX, and NOX. The glucoside group of quercetin 3'-glucoside plays an important role in determining the correct position in the attachment and supporting the formation of hydrogen bonds with receptors.

Hikmah et al. (2022) found vitamin E derivatives block TNF-α and activate NF-κB in human Jurkat T cells. RFO treatment can boost the production of anti-inflammatory cytokines like IL-10 and IL-22, reducing the activity of pro-inflammatory cytokines like TNF-α and IL-1. Lymphocyte proliferation can boost IL-10 and IL-22 production, inhibiting NF-κB activation and IL-1 and COX-2 production (Khiong et al. 2009). RFO also inhibits NF-κB activation through translocation to the nucleus and in vitro binding of active proteins to κB DNA (Hikmah et al. 2022). RFO inhibition may decrease iNOS by inhibiting NFKB. According to Sauqi et al. (2020), methyl palmitate in RFO can activate PPAR-g and downregulate the NF-κB pathway. Study Rhee et al. (2020) discovered that RFO suppresses NF-κB and ROS inflammatory responses by inhibiting iNOS expression, NO and PGE2 generation, and their underlying mechanisms. Genes including iNOS, COX-2, TNF-α, and IL-1β that have NF-κB binding motifs in their promoters will also be altered. We found that RFO may alleviate oxidative stress and inflammation-related illnesses. However, future research should examine oxidative stress indicators and other inflammatory mediators (COX-2, IL-1B, IL-10, IL-22, etc.) that may cause pain.

Limitation

The limitations of this study are that the number of animals used in this study is a limited number, yet sufficient for statistical analysis, and only focuses on male rats. Furthermore, the potential for variability in cold allodynia and thermal hyperalgesia tests such as environmental factors, animal handling, and animal pain perception thresholds greatly affects the results.

Conclusion

The findings demonstrated that STZ-induced DPN in rat spinal cord tissue increased oxidative stress and inflammation. RFO treatment at 0.6 kg/BW and 1.2 kg/BW, their endogenous antioxidant capacity is enhanced, tissue is protected against oxidative stress resulting from decreased lipid peroxidation, and cells are shielded against inflammation. Nevertheless, additional investigation is required to ascertain the therapeutic and preventative impacts of RFO via alternative pathways.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.
The authors declared that no experiments on humans or human tissues were performed for the present study.
The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.
Experiments on animals: KEP/I/2024/II/H050224IT/MDNS
The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

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Author contributions

Indah Tri Lestari: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Original draft. Kusnandar Anggadiredja: Project administration, Supervision, Original draft, Validation. Afrillia Nuryanti Garmana: Project administration, Supervision, Original draft, Validation.

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Data availability

All of the data that support the findings of this study are available in the main text.

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