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Nephroprotective Effect of Milkfish, Patin, and Snakehead Fish Oil by Suppressing Inflammation and Oxidative Stress in Diabetic Rats

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ABSTRACT

Diabetic nephropathy (DN) has been linked to a number of long-term problems caused by diabetes mellitus. Inflammatory and oxidative stress pathways contribute to DN development and progression. Many studies have shown the preventive advantages of diets rich in substances like anti-inflammatory and antioxidant elements like omega-3 fatty acids (n-3 FA) in preventing DN. Milkfish (*Chanos chanos* F.), patin (*Pangasius micronema* Blkr.), and snakehead fish (*Chana striata* Bloch) are fish oils which are known to contain n-3 FA. This study aims to evaluate the nephroprotective effect of three types of fish oils in a rat model of diabetes mellitus. Thirty male rats were used in this study. The animals were randomly divided into six groups (n = 5): the non-diabetic group, the diabetes mellitus group, the diabetic with 150 mg/kg metformin orally group, the diabetic with 1000 mg/kg milkfish oil orally group, the diabetic with 1000 mg/kg patin fish oil orally group, and the diabetic with 1000 mg/kg snakehead fish oil orally group. Diabetes models were induced using 65 mg/kg streptozotocin and 230 mg/kg nicotinamide intraperitoneally. The test was carried out for 8 weeks, followed by the observation of the biochemical profiles of blood, urine, oxidative stress, and the immunohistochemistry of the kidneys. A normally and homogeneously distributed test followed by a one-way analysis of variance (ANOVA) and the LSD post hoc test were used to analyse data at P 0.05. The results showed that serum creatinine levels did not differ significantly after the administration of milkfish, catfish, and snakehead fish oil for 8 weeks ($p \geq 0.05$). Different results were shown where the levels of serum BUN, uric acid, urine urea, and microalbumin urine were significantly different after administration of the three types of fish oil ($p \leq 0.05$). The same results were shown in oxidative stress (superoxide dismutase, glutathione, and malondialdehyde) and inflammation (interleukin-6 and tumor necrosis factor-α) profiles ($p \leq 0.05$).

Keywords: Fish oil, nephroprotective, inflammation, oxidative stress

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized by high blood sugar caused by problems with insulin release, insulin sensitivity, or both (AlFaris *et al,* 2020). It has been linked to a number of long-term problems, including retinopathy, neuropathy, and nephropathy (Bhatti *et al,* 2022). Diabetic nephropathy (DN) is one of the most serious consequences of diabetes mellitus, with morbidity and death rates increased in developed countries. If uncontrolled, 20-40% of diabetic people with microalbuminuria advance to overt nephropathy, and 20% develop end-stage renal failure (Gong *et al,* 2022; Kishore *et al,* 2017). Inflammatory pathways contribute to DN development and progression (Araújo *et al,* 2020). This process involves monocytes, macrophages, lymphocytes, chemokines, and cytokines (Araújo *et al,* 2016). Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) may contribute to the development of DN by causing clinical complications (Fathy *et al,*

2019). The endeavor to create remedies from natural sources remains a fascinating and challenging task. This includes the exploration of extracts (Nugroho *et al*. 2011a), fractions (Harwoko *et al*. 2012), and isolates (Nugroho *et al*. 2011b; Nugroho *et al*. 2011c) obtained from natural ingredients, as well as the measurement of their active components (Syukri *et al*., 2016). Moreover, many studies have shown the preventive advantages of diets like the Mediterranean diet, rich in anti-inflammatory and antioxidant elements like omega-3 fatty acids (n-3 FA), in preventing DN (Itsiopoulos *et al,* 2018; Usta *et al,* 2020).

Milkfish (*Chanos chanos* F.), patin (*Pangasius micronema* Blkr.), and snakehead fish (*Chana striata* Bloch) are a type of fish oil that is known to contain n-3 FA, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Hidayah *et al,* 2022; Putri *et al,* 2019; Sugata *et al,* 2019). The beneficial effects of n-3 FA rich fish oil on glycemic control are at least caused by the reduction of plasma adipokine disturbance, the improvement of insulin sensitivity through the maintenance of normal insulin signalling, the suppression of pro-inflammatory signaling, and the increase of AMPK activation in skeletal muscle (Keapai *et al,* 2016). Furthermore, n-3 FA was associated with a moderate nephroprotective effect by reducing oxidative stress and inflammation in renal tissue (El-Boshy *et al,* 2021). Long-chain n-3FA controls inflammatory pathways by competing with the enzymatic metabolism of arachidonic acid, which is transformed into pro-inflammatory eicosanoids (Han *et al,* 2016).

Fish oil has been demonstrated to have therapeutic benefits in the treatment of IgA nephropathy (Donadio *et al,* 1999). Shapiro *et al*. (2011) reported that the use of fish oil has a beneficial effect on albuminuria, hypertension, and dyslipidemia in patients with insulin-dependent diabetes mellitus (IDDM) with diabetic nephropathy. The effect of reducing albuminuria and maintaining kidney function in DM patients depends on the doses used (Han *et al,* 2016). However, there is limited information regarding the effect of milkfish, patin, and snakehead fish oil rich in n-3 FA on its nephroprotective effect in DM. The novelty aspect of this study is that it looks into the effects of fish oils from milkfish, patin, and snakehead fish, which are thought to protect the kidneys through an anti-inflammatory and oxidative stress pathway. In diagnosing the progression of DN, the immunohistochemical approach is considered an accurate method (Araújo *et al,* 2020; Haligur *et al,* 2012). In this study, the biochemical profile of kidney function in the blood and urine will be looked at, and an immunohistochemical approach will also be used to look at the inflammatory profile of the kidney organ.

MATERIALS AND METHODS

Milkfish and patin sample from the Juwana Pati traditional market in Central Java, Indonesia. Snakehead fish were purchased from local farmers in Kendal, Central Java, Indonesia. Fish authenticated by the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, with identification numbers 21.15.2/UN1/FFA.2/S1/PT/2022. Chemicals such as streptozotocin (STZ) and nicotinamide (NIC) were purchased from Sigma Aldrich USA. Reagents of blood urea nitrogen (BUN), creatinine, and uric acid were purchased from BioSystem, Germany. Kit reagent superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) were purchased from FineTest China.

Preparation of fish oil

Milkfish, patin, and snakehead fish are cleaned, sorted into flesh and intestines, and then cut into little pieces. Separately, the fish are extracted using the wet rendering method for 15 minutes at 80-95 ^{o}C. Using a separatory funnel, the extracted liquid is split into the oil and water phases. Before being refined, the crude fish oil is stored in a dark bottle at 2-8 ^{o}C (Sasongko *et al,* 2017). The crude fish oil was then further processed to obtain pure fish oil. This purification process includes stages of the heating process, the addition of adsorbents, and vacuum filtration. The bleaching process is carried out by adding an adsorbent (bentonite) of 1% by weight of the oil when the temperature reaches 55–60 ^{o}C. Then the heating is continued until it reaches a temperature of 80 ºC for 30 minutes. Furthermore, the oil is filtered using a vacuum filter, and the weight of the resulting oil is weighed as the yield of pure milkfish oil (Nadhiro *et al,* 2018).

Animals and experimental design

Wistar rats weighing 160–200 g was used for this experiment. Animals were maintained in a temperature-controlled room with a 12 h light-dark cycle and were given a standard laboratory diet with an unlimited supply of drinking water.

The experiments were carried out after approval by the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Indonesia (Approval No: 00034/04/LPPT/VIII/2021). The animals were randomly divided into six groups (n=5): non-diabetic group (ND), diabetes mellitus group (DM), diabetic with 150 mg/kg metformin orally group (DM-MET), diabetic with 1000 mg/kg milkfish oil orally (DM-MFO), diabetic with 1000 mg/kg patin fish oil orally (DM-PFO), and diabetic with 1000 mg/kg snakehead fish oil orally (DM-SFO).

Diabetes mellitus animal models

The Wistar rat was induced using 65 mg/kg streptozotocin (STZ) in citrate buffer (0.1 M, pH 4.5) i.p and 230 mg/kg nicotinamide (NIC) i.p 15 minutes after STZ induction. After 7 days of STZ-NIC injection, rats with a fasting plasma glucose level of ≥ 250 mg/dL were used in this study. Experiments and observations were carried out for 8 weeks (Sasongko *et al,* 2022).

Biochemical analysis

Blood and urine were collected at the end of the study for the measurement of biochemical profiles such as creatinine (Cr), blood urea nitrogen (BUN), uric acid (UA), and microalbumin. Protocol for measuring according to the kit reagent BioSystem manufacturer in Germany. The rats were euthanized with xylazine (5 mg/kg) and ketamine HCl (40 mg/kg). The kidney organs were excised for IL-6 and TNF-α scoring by immunohistochemistry (IHC).

Measurement of oxidative stress in renal organ

The renal oxidative stress activities of superoxide dismutase, glutathione, and malondialdehyde were measured using kits, according to the FineTest China manufacturer's instructions.

Immunohistochemistry

The immunohistochemistry of the renal profile was measured using kits, according to the manufacturer's instructions (Abcam UK). The expression profile of inflammatory cytokines and chemokines analyzed is interleukin-6 (IL-6) dan tumor necrosis factor-α (TNF-α).

Statistical analysis

The data were analyzed using SPSS-18 software and one-way analysis of variance (ANOVA) followed by the LSD post hoc test. The data were shown as mean SEM. At $p \leq 0.05$, data were considered statistically significant.

RESULTS AND DISCUSSION

In this study, streptozotocin-nicotinamide induction was able to cause diabetes mellitus in rats (Sasongko *et al,* 2022). The effect of the administration of milkfish, catfish, and snakehead fish oil for 8 weeks on the biochemical profile of the blood and urine (Table I). Serum creatinine levels showed no significant increase after eight weeks of the experiment. However, different urine profiles showed significant differences in each group ($p \leq 0.05$). After 8 weeks, the rats were diagnosed with DM, and there was a decrease in creatinine levels in the urine. There was a significant difference between the non-diabetic group and the diabetes group ($p \leq 0.05$). This shows that the DM condition affects the rat's kidney function, though there is no significant change in the blood creatinine profile. This difference depends on the severity of kidney damage (Iseki *et al,* 1997; Nugent *et al,* 2021). Generally, serum creatinine levels experience a significant increase when it is at the stage of end-stage renal disease (Aryaie *et al,* 2022; Cohen *et al,* 2019). In the group of rats that controlled their blood glucose using metformin, there appeared to be a significant difference in their urine creatinine levels compared to the diabetes group. Blood glucose management is a treatment goal for DN prevention (Samsu, 2021). In the group given fish oil, only snakehead fish oil showed improvement in kidney function, where the urine creatinine level had a significant increase ($p \leq 0.05$). While the group given milkfish and patin fish oil did not show any significant changes compared to the group of diabetic rats ($p \geq 0.05$). In the BUN, uric acid, and urea urine parameters, the biochemical profiles showed more changes after the administration of milkfish, patin, and snakehead fish oil samples. There was a significant difference after the administration of the three types of fish oil ($p \leq 0.05$) (Table I). Although BUN, uric acid, and urine urea are not specific parameters, they can provide an overview of kidney function (Guo *et al,* 2021; Rahmani *et al,* 2022). Microalbumin in urine is a specific marker of kidney damage (Tsai *et al,* 2021). The diabetic rat group contained significantly different amounts of urine microalbumin compared to the non-diabetic rat group ($p \leq 0.05$) (Table I). Microalbumin levels decreased significantly after administration of milkfish, catfish, and snakehead fish oil.

Table I. The effect of fish oil administration on the blood and urine biochemical profiles of rats at the end of the experiment (n = 5). (*), There is a significant difference with the normal group (p≤0.05); (#), There was a significant difference with the negative control group (p≤0.05).

Group	Serum			Urine		
	Creatinine (mg/dL)	Blood Urea Nitrogen (BUN) (mg/dL)	Uric acid (mg/dL)	Creatinine (mg/dL)	Urea (mg/dL)	Microalbumine (mg/L)
ND	0.72±0.05	57.20±5.82#	2.82±0.09#	2.22±0.46#	66.64±11.25#	-0.49 ± 0.08#
DM	0.74±0.02	434.80±52.52*	3.54±0.34*	0.46± 0.12*	13.07±2.13*	0.97 ± 0.15*
DM-MET	0.70±0.02	98.00±17.37*#	3.08±0.26	0.72±0.11*#	52.93±3.60#	-0.22 ± 0.32#
DM-MFO	0.62±0.02	98.20±4.14*#	2.82±0.10#	0.20±0.05*	35.14±8.34#	-0.10 ± 0.32#
DM-PFO	0.68±0.04	135.60±11.67*#	2.95±0.22#	0.52±0.08*	34.71±4.06#	-0.42 ± 0.21#
DM-SFO	0.62±0.02	109.80±14.04*#	2.95±0.08#	1.69±0.16#	54.64±8.28#	0.05 ± 0.46#

ND: non-diabetic group, DM: diabetes mellitus group, DM-MET: diabetic with 150 mg/kg metformin orally group, DM-MFO: diabetic with 1000 mg/kg milkfish oil orally group, DM-PFO: diabetic with 1000 mg/kg patin fish oil orally group, DM-SFO : diabetic with 1000 mg/kg snakehead fish oil orally group.

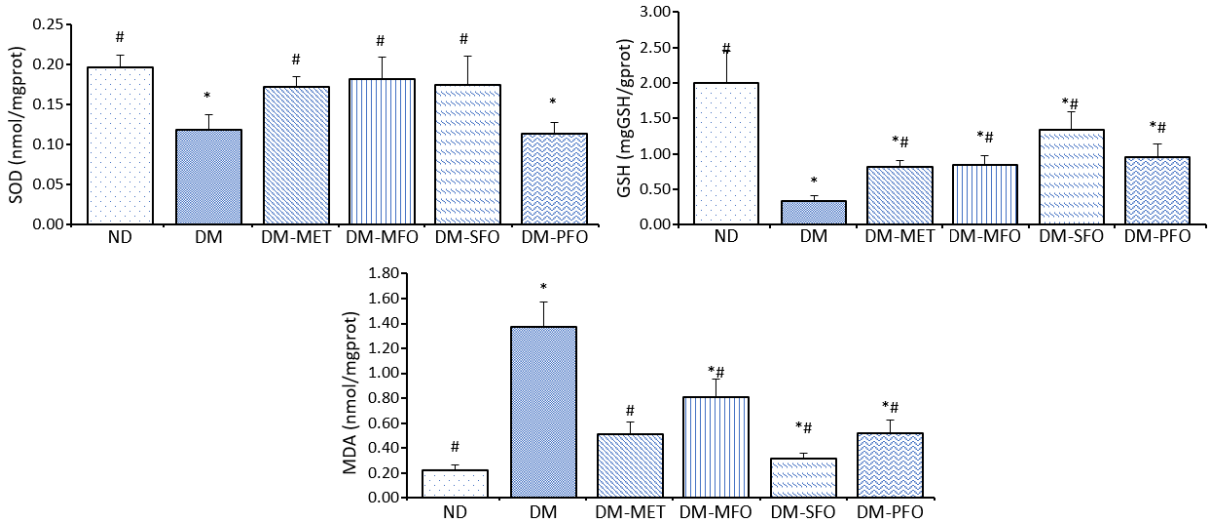

Figure 1. Effect of fish oil administration on SOD, GSH, and MDA levels in rat kidneys at the end of the experiment (n = 5). (*) There is a significant difference with the normal group (p≤0.05); (#) There was a significant difference with the negative control group (p≤0.05). ND: non-diabetic group, DM: diabetes mellitus group, DM-MET: diabetic with 150 mg/kg metformin orally group, DM-MFO: diabetic with 1000 mg/kg milkfish oil orally group, DM-PFO: diabetic with 1000 mg/kg patin fish oil orally group, DM-SFO : diabetic with 1000 mg/kg snakehead fish oil orally group.

The oxidative stress profile of rat kidney organs after eight weeks of the experiment (Figure 1). Superoxide dismutase (SOD) and glutathione (GSH) levels in the diabetic rat group decreased significantly when compared to the non-diabetic group (p≤0.05). Meanwhile, the MDA level in the diabetic rat group experienced a significant increase. This shows that the DM condition can increase oxidative stress, as marked by a decrease in SOD and GSH levels and an increase in MDA levels. Meanwhile, the group of rats whose blood glucose levels were controlled using metformin showed an increase in SOD and GSH levels and a decrease in MDA levels compared to the diabetes group (p≤0.05). In diabetic rats that were given milkfish, patin, and snakehead fish oil for 8 weeks, there was a significant increase in GSH levels and anincrease in MDA (Figure 1). Meanwhile, SOD levels increased significantly after the administration of milkfish and snakehead oil (p≤0.05). High blood glucose levels can increase free radicals.

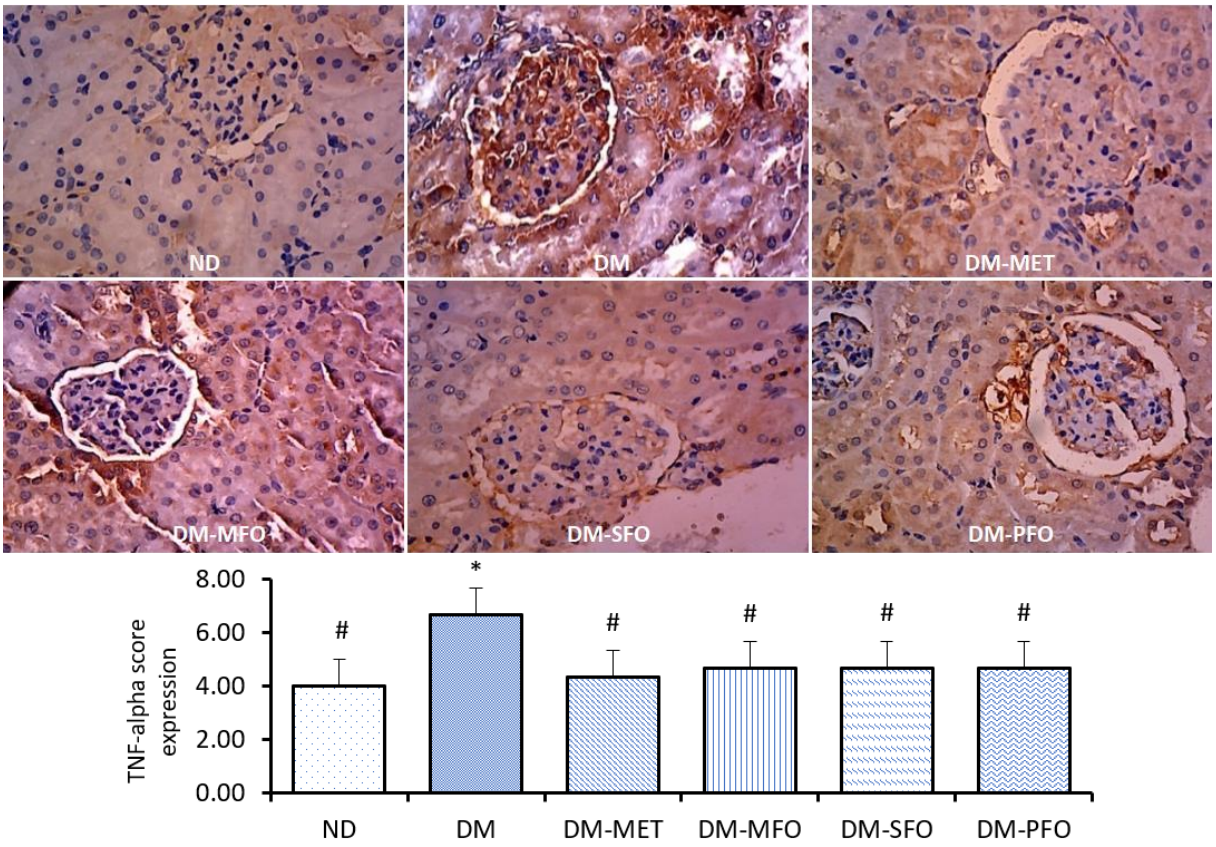

Figure 2. TNF-α cytokines expression on immunohistochemical analysis of rat kidney organs. *, Significantly different compared to the non-diabetic group ($p<0.05$). #, Significantly different compared to the diabetes mellitus group ($p\leq0.05$). ND: non-diabetic group, DM: diabetes mellitus group, DM-MET: diabetic with 150 mg/kg metformin orally group, DM-MFO: diabetic with 1000 mg/kg milkfish oil orally group, DM-PFO: diabetic with 1000 mg/kg patin fish oil orally group, DM-SFO : diabetic with 1000 mg/kg snakehead fish oil orally group.

MDA is the end product of free radicals' peroxidation of lipids (Sasongko *et al,* 2018). The intensity of an organism's oxidative stress response is proportional to the concentration of MDA in vivo (Szeto, 2006). SOD and GSH are both essential antioxidant indicators (Liu *et al,* 2020). Superoxide is converted by SOD into oxygen and hydrogen peroxide. As part of the cellular response to oxidative stress, GSH scavenges oxidants (Yoo *et al,* 2019). It is considered that an imbalance in the generation of reactive oxygen species and antioxidants contributes to diabetes-related renal failure (Singh *et al,* 2020). While antioxidant enzymes are stimulated to protect cells and tissues from harm (Sayed, 2012), an increasing number of animal tests and clinical research indicate that diabetes may interfere with the antioxidant defense system by modulating antioxidant enzyme activity (Sathibabu Uddandrao *et al,* 2019).

Immunohistochemical scoring on TNF-α and IL-6 parameters, after 8 weeks of the experiment, the kidney organs of the DM group showed inflammation as indicated by significantly increased expression of TNF-α and IL-6 cytokines ($p\leq0.05$) (Figure 2 and 3). This suggests that chronic diabetes in rats results in injury to the kidney, which is characterized by an increased inflammatory response. These results are confirmed by the biochemical profiles of blood (BUN and UA) and urine (Cr, urea, and microalbumin) in Table 1. The group of rats whose blood glucose was controlled with metformin showed a significantly different decrease in inflammation compared to the DM group ($p\leq0.05$).

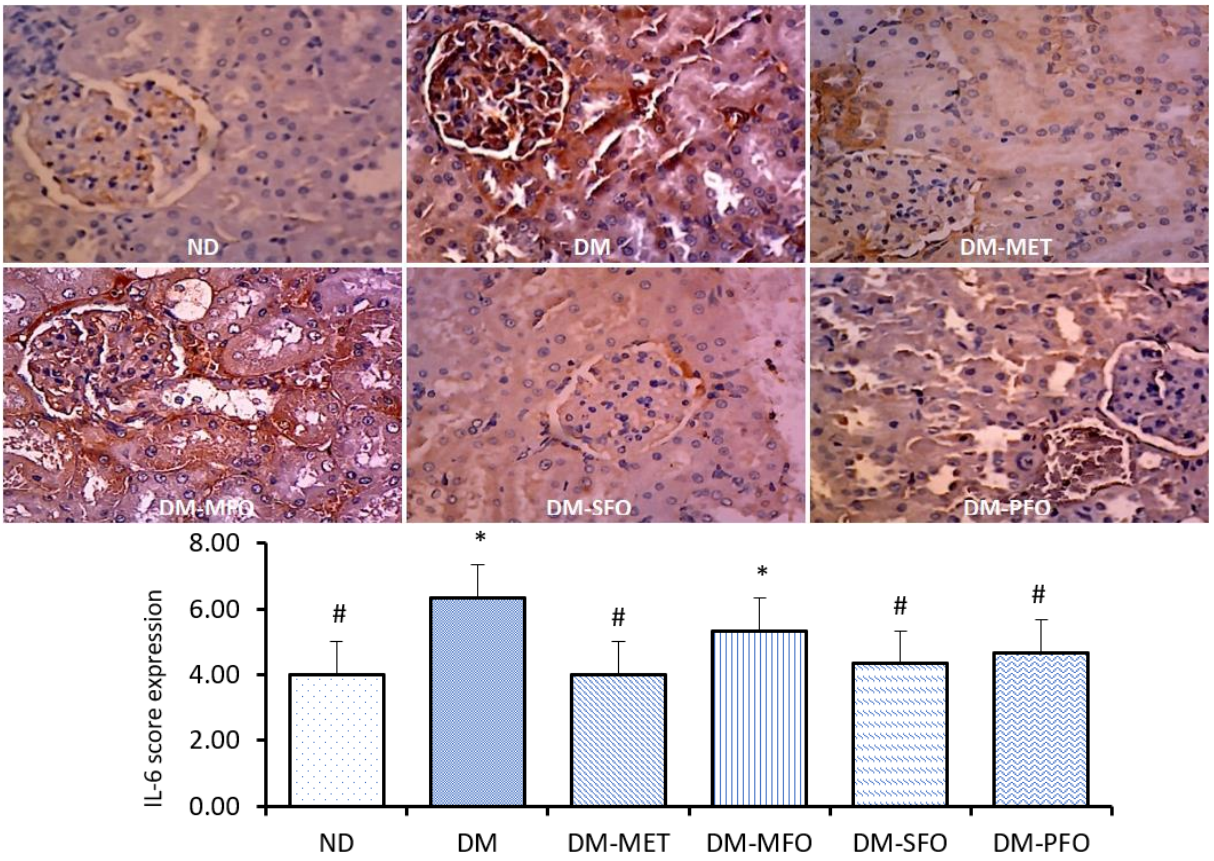

Figure 3. IL-6 cytokines expression on immunohistochemical analysis of rat kidney organs. *, Significantly different compared to the non-diabetic group (p≤0.05). #, Significantly different compared to the diabetes mellitus group (p≤0.05). ND: non-diabetic group, DM: diabetes mellitus group, DM-MET: diabetic with 150 mg/kg metformin orally group, DM-MFO: diabetic with 1000 mg/kg milkfish oil orally group, DM-PFO: diabetic with 1000 mg/kg patin fish oil orally group, DM-SFO : diabetic with 1000 mg/kg snakehead fish oil orally group.

Meanwhile, rats given fish oil showed a significant decrease in inflammatory expression in TNF-α and IL-6 parameters (p≤0.05), except for the milkfish oil group with IL-6 parameters (p≥0.05). The same results were also seen in the oxidative stress profile (Figure 1) after the administration of milkfish, patin, and snakehead fish oils. Though Figures 2 and 3 don't show much difference between the three types of fish oil, they can still reduce inflammation in the kidneys.

This research investigated the protective effects of milkfish, patin, and snakehead fish oils against DN in terms of metabolic control, oxidative stress, and inflammation. Previously to therapeutic initiation, DN was confirmed by elevated BUN, UA, and albuminuria and decreased urine Cr and urea levels compared to the DM group. Compared to the DM group, the oxidative stress (MDA) and inflammatory (TNF- & IL6) markers were elevated in the renal tissues, while the anti-oxidant (GSH and SOD) enzymes decreased. Many studies have characterized the diagnosis of DN including persistent albuminuria (30-300 mg/day) (Persson & Rossing, 2018), glomerular hyperfiltration to the level of end-stage renal disease (Sagoo & Gnudi, 2018). Brosius *et al*. (2009) said that the criteria for a progressive rat model of diabetic nephropathy increased albuminuria by more than 10-fold compared to controls for that strain at the same age and sex. The test results are appropriate where the urine microalbumin level (Table I) is more than 10-fold. Generally, microalbuminuria conditions in humans develop progressively into macroalbuminuria, occurring 10–15 years after the

diagnosis of diabetes mellitus (Allan *et al,* 2023). The n-3 FA in fish oils is reported to have anti-diabetic, anti-oxidative stress, and anti-inflammatory effects that reduce albuminuria and slow the progression of DN in rats (de Assis *et al,* 2015; Vitlov Uljević *et al,* 2019). The inflammatory markers did not show any differences between types of fish oil after being given for 8 weeks. However, snakehead fish oil showed improvement in oxidative stress. Although the n-3 FA content of snakehead fish oil is reported to be higher, especially EPA (Putri *et al,* 2019; Sasongko *et al,* 2022) than milkfish and catfish oils, a more comprehensive study is needed. The quality of fish oil affects the content of n-3 FA, especially EPA and DHA, which play an active role in providing therapeutic effects (Bonilla-Méndez *et al,* 2018). Fish oil contains EPA and DHA, with EPA being more abundant. Several studies suggest that EPA and DHA affect health outcomes differently, especially cardiometabolic (Ghasemi Fard *et al,* 2019). Mozaffarian & Wu (2012) reported that the n-3 FA biological pathway plays a role in regulating the function of cell membranes and organelles, initiating ion channels and cellular electrophysiology, regulating nuclear receptors and transcription factors, and producing bioactive metabolites derived from n-3 FA.

CONCLUSION

Milkfish, patin, and snakehead fish oils in single therapies exhibited limited glycaemic control and were associated with moderate nephroprotection by suppressing inflammation and oxidative stress. Nevertheless, this research did not assess the effects of fish oil on renal hemodynamics, GFR, or glucose and lipid homeostasis molecular pathways. Hence, further study is needed to figure out how milkfish, patin, and snakehead fish oil affect renal blood flow, glomerular and tubular functioning, and metabolic pathways.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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