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Original Article

Randomized controlled trial on the regulation of MMP-9-NGAL ratio in diabetic patients with early renal dysfunction by *Piper crocatum* Ruiz & Pav. functional foods

[Ensayo controlado aleatorizado sobre la regulación del ratio MMP-9-NGAL en pacientes diabéticos con disfunción renal temprana por alimentos funcionales de *Piper crocatum* Ruiz & Pav.]

Andina Setyawati[1*], Nuurhidayat Jafar[2], Muhammad Raihan[3], Heri Kristianto[4]

[1]Department of Surgical and Medical Nursing, Faculty of Nursing, Hasanuddin University, Jl. Perintis Kemerdekaan km 10, Kampus Tamalanrea, Makassar, 90245, Indonesia.
[2]Department of Community Nursing, Faculty of Nursing, Hasanuddin University, Jl. Perintis Kemerdekaan km 10, Kampus Tamalanrea, Makassar, 90245, Indonesia.
[3]Faculty of Pharmacy, Hasanuddin University, Jl. Perintis Kemerdekaan km 10, Kampus Tamalanrea, Makassar, 90245, Indonesia.
[4]Department of Medical and Surgical Nursing, Faculty of Health Science, University of Brawijaya, Jl. Veteran, Lowokwaru, Malang, Jawa Timur, 65145, Indonesia.
*E-mail: andina@unhas.ac.id

Abstract

Context: Diabetic nephropathy (DN) remains a leading cause of end-stage renal disease, largely driven by inflammation and oxidative stress. *Piper crocatum* is rich in bioactive compounds with antioxidant and anti-inflammatory effects.

Aims: To evaluate the effects of *P. crocatum*-enriched functional foods on renal biomarkers in diabetic patients with early renal dysfunction.

Methods: A 12-week double-blind, randomized, placebo-controlled trial was conducted among 387 diabetic adults with mild renal impairment. Participants consumed either *P. crocatum*-enriched cookies or placebo cookies. Serum matrix metalloproteinase-9 (MMP-9), neutrophil gelatinase-associated lipocalin (NGAL), and the MMP-9-NGAL ratio were measured pre- and post-intervention.

Results: Compared with placebo, *P. crocatum* significantly reduced MMP-9 (518.45 to 350.34 ng/mL, false discovery rate (FDR)-adjusted p=0.0003), NGAL (195.67 to 174.31 ng/mL, FDR-adjusted p=0.0016), and the MMP-9-NGAL ratio (2.65 to 1.99, FDR-adjusted p=0.0011). Effect sizes were large. The placebo showed minimal changes.

Conclusions: *P. crocatum* supplementation improved renal inflammation and oxidative stress markers, suggesting its potential as an adjunct therapy for DN.

Keywords: clinical trial; diabetes mellitus; functional food; matrix metalloproteinase-9; neutrophil gelatinase-associated lipocalin; renal insufficiency.

Resumen

Contexto: La nefropatía diabética (ND) sigue siendo una de las principales causas de enfermedad renal terminal, impulsada principalmente por la inflamación y el estrés oxidativo. *Piper crocatum* es rico en compuestos bioactivos con efectos antioxidantes y antiinflamatorios.

Objetivos: Evaluar los efectos de los alimentos funcionales enriquecidos con *P. crocatum* sobre los biomarcadores renales en pacientes diabéticos con disfunción renal temprana.

Métodos: Se realizó un ensayo doble ciego, aleatorizado y controlado con placebo de 12 semanas de duración en 387 adultos diabéticos con insuficiencia renal leve. Los participantes consumieron galletas enriquecidas con *P. crocatum* o galletas placebo. Se midieron la metaloproteinasa de matriz sérica 9 (MMP-9), la lipocalina asociada a la gelatinasa de neutrófilos (NGAL) y el cociente MMP-9-NGAL antes y después de la intervención.

Resultados: En comparación con placebo, *P. crocatum* redujo significativamente la MMP-9 (518,45 a 350,34 ng/mL, p ajustada por FDR = 0,0003), la NGAL (195,67 a 174,31 ng/mL, p ajustada por tasa de falsos descubrimientos (FDR) = 0,0016) y la relación MMP-9-NGAL (2,65 a 1,99, p ajustada por FDR = 0,0011). La magnitud del efecto fue amplia. El placebo mostró cambios mínimos.

Conclusiones: La suplementación con *P. crocatum* mejoró los marcadores de inflamación renal y estrés oxidativo, lo que sugiere su potencial como terapia complementaria para la ND.

Palabras Clave: alimento funcional; diabetes mellitus; ensayo clínico; insuficiencia renal; lipocalina asociada a la gelatinasa de neutrófilos; metaloproteinasa de matriz-9.

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AUTHOR INFO
ORCID: 0000-0003-4728-0815 (AS)
0000-0002-7715-8805 (NJ)
0000-0002-8031-5445 (MR)
0000-0002-9869-4519 (HK)

Abbreviations: DM: Diabetes mellitus; DN: Diabetic nephropathy; ECM: Extracellular matrix; eGFR: Estimated glomerular filtration rate; ESRD: End-stage renal disease; FDR: False discovery rate; MMP-9: Matrix metalloproteinase-9; NGAL: Neutrophil gelatinase-associated lipocalin; qPCR: Quantitative polymerase chain reaction; ROS: Reactive oxygen species; SOD1: Superoxide dismutase 1.

INTRODUCTION

Diabetic nephropathy (DN) is a kidney complication that occurs in approximately 30–40% of patients with diabetes mellitus (DM), and it remains one of the leading causes of end-stage renal disease (ESRD) worldwide (American Diabetes Association, 2021). As diabetes prevalence continues to rise globally, DN is expected to become an even greater health and economic burden, particularly in resource-limited settings where access to dialysis and kidney transplantation is limited (Fletcher et al., 2022; International Diabetes Federation, 2021). DN develops gradually due to prolonged high blood sugar levels, which trigger chronic inflammation and oxidative stress, an imbalance between free radicals and antioxidants in the body (Agarwal, 2021). With global diabetes prevalence projected to rise from 537 million in 2021 to 783 million by 2045, the burden of DN will increase substantially (Powers et al., 2020). These processes damage kidney tissues, leading to a decline in glomerular (filtering unit) and tubular (reabsorption unit) function over time. Current treatment strategies focus primarily on controlling blood sugar and blood pressure but do not directly target the underlying inflammatory and oxidative pathways responsible for kidney damage (Jagdale et al., 2019; Kumar et al., 2023).

In recent years, natural plant-based compounds, especially antioxidants and anti-inflammatory agents, have gained interest as potential complementary therapies for DN. One such plant is *Piper crocatum* (red betel leaf), a traditional Indonesian medicinal herb rich in polyphenols and flavonoids, which are naturally occurring compounds known to neutralize free radicals and reduce inflammation. In addition to polyphenols and flavonoids, *P. crocatum* contains glycosidic compounds, such as flavone glycosides and phenolic glycosides, which may further enhance its bioactivity in modulating oxidative pathways (Alsawaf et al., 2022; Krawczyk et al., 2023; Setyawati et al., 2021). These properties make it a potential adjunct therapy for slowing nephropathy progression and improving outcomes. Matrix metalloproteinase-9 (MMP-9), an enzyme linked to inflammation-driven extracellular matrix (ECM) degradation and glomerular damage, reflects the inflammatory processes driving fibrosis and renal injury, highlighting its role in disease progression and severity (Jin et al., 2023). Laboratory studies suggest that extracts from *P. crocatum* can influence cellular defense mechanisms and improve tissue repair in conditions associated with oxidative damage (Serra et al., 2016).

The bioactive compounds in *P. crocatum* are believed to mitigate oxidative stress and inflammatory signaling pathways, which are primary drivers of MMP-9 upregulation (Latronico et al., 2024). Specifically, *P. crocatum* has been shown to influence pathways involving p53 and SOD1, proteins that regulate cellular response to oxidative stress and may indirectly reduce MMP-9 levels by stabilizing ECM components and inhibiting excessive matrix breakdown (He et al., 2025). Similarly, the antioxidative action of *P. crocatum* may protect against tubular cell injury, thereby reducing neutrophil gelatinase-associated lipocalin (NGAL) levels (St-Pierre, 2021). By modulating both MMP-9 and NGAL, *P. crocatum* could help restore the MMP-9-NGAL balance, presenting a promising adjunct therapy to reduce renal inflammation and delay DN progression. The MMP-9-NGAL ratio serves as a composite biomarker that integrates markers of ECM degradation (MMP-9) and tubular injury (NGAL), offering a more comprehensive indication of renal inflammation and early damage in DN (Kostopoulou et al., 2024; Yang et al., 2021).

Several international studies have highlighted the therapeutic potential of phytochemicals in managing DN. For example, compounds derived from *Centella asiatica*, *Curcuma longa*, *Berberis aristata*, and *Aloe vera* have shown promising anti-inflammatory, antioxidant, and nephroprotective effects in preclinical and clinical models of DN (Dubey et al., 2024; Muñiz-Ramirez et al., 2020; Ovaditya et al., 2022; Oyenihi et al., 2020). These findings support the rationale for investigating *P. crocatum* as a potential herbal intervention for modulating key renal biomarkers such as MMP-9 and NGAL.

Despite promising preclinical evidence of its antioxidant activity, the effects of *P. crocatum* on clinical markers of kidney inflammation and injury in DN have not been evaluated in human trials. In particular, key blood biomarkers such as MMP-9 and NGAL (a protein that signals early kidney injury) are known to be elevated in DN patients (Dahiya et al., 2023; Provenzano et al., 2020), but whether *P. crocatum* influences their levels remains unclear.

This study aims to examine the effect of a *P. crocatum*-based functional food on MMP-9, NGAL, and their ratio in patients with type 2 diabetes and early kidney dysfunction. Accordingly, this research provides early clinical evidence on the potential of *P. crocatum* as an adjunct intervention to reduce renal inflammation and improve kidney outcomes in DN. To our knowledge, this is the first randomized controlled trial

evaluating the clinical effects of *P. crocatum* on renal inflammation and injury biomarkers in human subjects with early DN, thus filling a critical gap in the current literature dominated by preclinical studies.

MATERIAL AND METHODS

Chemicals and reagents

All chemicals used in this study were of analytical grade. ELISA kits for MMP-9 (Cat# ab214572) and NGAL (Cat# ab119605) were purchased from Abcam® (Cambridge, UK). The reagents used for serum sample preparation, including phosphate-buffered saline (PBS) and protease inhibitors, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Food-grade ethanol (96%) was used for the extraction process, purchased from Merck® (Darmstadt, Germany).

The bioactive-rich cookie formulation was prepared using *P. crocatum* extracts. These extracts were produced through a maceration process using food-grade ethanol (96%) followed by filtration and concentration using a rotary evaporator (Buchi R-210, Buchi Labortechnik AG, Switzerland). The concentrated extract underwent fractionation using food-safe solvents, virgin coconut oil (VCO El Medinah, P-IRT NO. 2073402011960-20), chosen for their suitability in edible products. The fraction containing the highest antioxidant and anti-inflammatory activity, as determined by Setyawati et al. (2024), was selected for the cookie formulation. Active ingredients, including *P. crocatum* extracts and golden rice flour, were obtained from Herbal Indonesia Co. Ltd. (Jakarta, Indonesia), while the basic ingredients for cookie preparation were sourced from Bogasari® Flour Mills (Jakarta, Indonesia).

The cookie dough was prepared by mixing the active ingredients with the basic components and baked following the protocol outlined by Setyawati et al. (2024). The baking process was conducted at 158°C for 20 minutes to ensure the stability of the bioactive compounds in *P. crocatum*.

Plant material

Fresh leaves of *Piper crocatum* Ruiz & Pav. were collected from the Medicinal Plant Garden, Faculty of Pharmacy, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia (GPS coordinates: 7°46'28.6"S, 110°22'38.7"E) in June 2023. The plant material was identified and authenticated by a pharmacognosist from the Faculty of Pharmacy, Universitas Gadjah Mada. A voucher specimen (No. PC-UGM-2023-01) was deposited at the UGM Herbarium, Department of Pharmaceutical Biology. The part of the plant used was mature leaves, which were washed, shade-dried at room temperature, and ground into powder before ethanol extraction.

Study design and participants

This two-arm, parallel-group, randomized, placebo-controlled trial was conducted at a public health center in Makassar, Indonesia. Approved by the Hasanuddin University Faculty of Nursing Ethics Committee (No. 1220/UN4.14.1/TP.01.02/2024), all participants provided written informed consent. The trial is registered at ClinicalTrials.gov (NCT06676124) and adheres to the SPIRIT 2013 checklist.

Inclusion and exclusion criteria

A total of 403 diabetic patients were screened, and 387 participants met the inclusion criteria: Age above 20 years, diagnosis of DM, serum urea levels greater than 43 mg/dL, or serum creatinine levels greater than 0.90 mg/dL. Exclusion criteria included pregnancy, breastfeeding, other medical conditions requiring intensive care, and ongoing therapy for hypoalbuminemia. These 387 eligible participants were then randomized into the intervention group (n = 194) and placebo group (n = 193), as shown in the CONSORT flow diagram (Fig. 1). During the 12-week intervention period, three participants in the intervention group (one lost to follow-up and two who discontinued supplementation) and four in the placebo group (three lost to follow-up and one who discontinued supplementation) did not complete the study, resulting in 191 and 189 participants completing the intervention and placebo arms, respectively.

Adherence to the intervention was high, with 92.3% of participants in the intervention group and 91.5% in the placebo group consuming ≥80% of the prescribed cookies, as detailed in Table 1.

Table 1. Adherence and dropout summary.

Group	Allocated	Completed	Dropouts (n)	Reason for Dropout	Adherence ≥80%
Intervention	194	191	3	1 lost to follow-up, 2 discontinued voluntarily	92.3% (176/191)
Placebo	193	189	4	3 lost to follow-up, 1 discontinued voluntarily	91.5% (173/189)
Total	387	380	7		

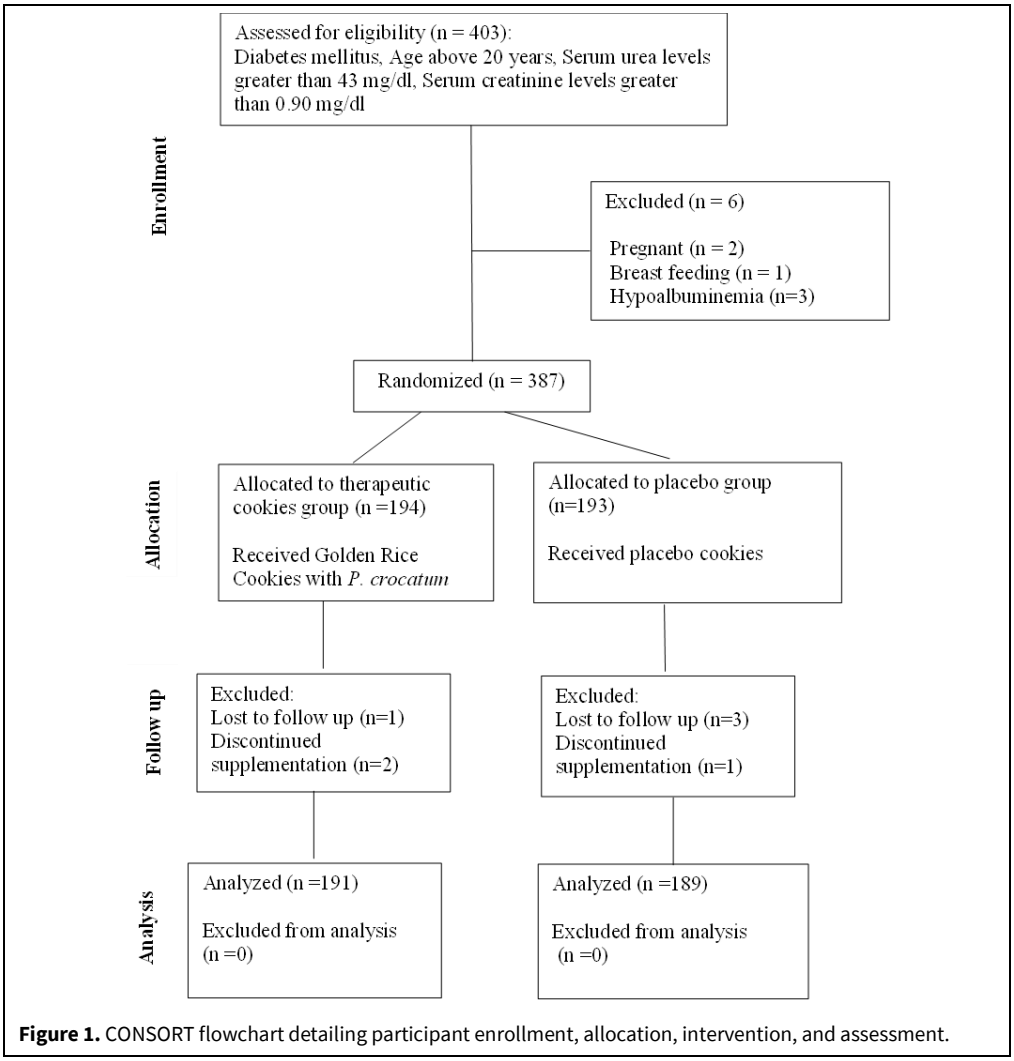

Figure 1. CONSORT flowchart detailing participant enrollment, allocation, intervention, and assessment.

Sample size determination

The sample size was calculated using G*Power software (version 3.1.9.2, Heinrich Heine University, Düsseldorf, Germany) to achieve a power of 90% at a 5% significance level (α = 0.05, two-tailed). An effect size of 0.6 was chosen based on previous studies evaluating changes in MMP-9 levels (Jones et al., 2019). The calculation assumed a standard deviation of 1.2 and a minimum detectable difference of 0.5 units between the intervention and control groups. This resulted in an estimated sample size of 155 participants per group. To accommodate a potential dropout rate of 20%, the final sample size was adjusted to 193 participants per group, ensuring adequate power to detect significant differences in the primary outcome variable.

Randomization and blinding

Participants were randomly assigned in a 1:1 ratio to either the intervention group (n = 194) or the placebo group (n = 193) using a block randomization method. An independent researcher conducted the randomization using a random number table, and group allocations were concealed in sealed opaque envelopes. The study was double-blinded; both participants and researchers were unaware of group assignments. The cookies were packaged identically and labeled with unique codes by the manufacturer (Healthy Snacks Co., Ltd., Makassar, Indonesia). To assess the effectiveness of blinding, participants were asked post-intervention to indicate whether they believed they had received the functional food intervention or the control product. The majority of responses (78%) indicated uncertainty or incorrect guesses, suggesting that participant blinding was maintained throughout the study.

Intervention

Bioactive-rich cookies containing *P. crocatum* extracts were administered to the intervention group, while cookies with identical composition but without active ingredients were administered to the placebo group. A dosage of two cookies per day was prescribed for participants (each containing 51 calories) for 12 weeks. The cookies were formulated to be low in glucose, containing 1% carbohydrates, 6% fat, 4% cholesterol, and 4% fiber. Each intervention cookie contained 10% of the standardized fraction of *P. crocatum*, which

has demonstrated modulatory effects on biomarkers associated with diabetic complications in prior pilot testing. This 10% concentration was selected to optimize therapeutic efficacy while preserving organoleptic acceptability, based on preliminary evaluation of both biomarker response and sensory tolerability.

High-performance liquid chromatography (HPLC) analysis confirmed that key glycosidic polyphenols in *P. crocatum*, including rutin (quercetin-3-O-rutinoside) and quercetin-3-O-glucoside (isoquercitrin), remained stable after baking. The retention rates were 92.4% and 89.7%, respectively, suggesting high thermal stability during cookie preparation.

Sensory evaluation of the *P. crocatum* functional cookies was conducted using a 5-point hedonic scale by both expert evaluators and participants. The cookies were rated positively for color and texture (experts: 4.00–4.10; patients: 4.05), while aroma and taste received slightly lower scores due to the inherent bitterness of the herbal extract.

To minimize potential bias arising from taste differences, the placebo cookies were formulated with a mild bitterness using natural food-grade bittering agents, ensuring both versions had comparable taste profiles. A pre-trial sensory test with 50 untrained panelists revealed no significant differences in taste, aroma, or texture between the placebo and intervention products ($p>0.05$). All cookies were identically shaped, colored, and packaged, and labeling was neutral to support blinding. Post-trial feedback also confirmed that participants were unable to distinguish between the two cookie types, supporting adherence and masking integrity.

Participant compliance was monitored through a returned product count method. At each biweekly follow-up. Any unused cookies were returned by participants, and adherence was calculated as the percentage of consumed units relative to the prescribed amount. Participants with consumption ≥80% were classified as adherent. Based on this approach, 92.3% of participants in the intervention group were deemed adherent. Participants were instructed to report any discomfort or adverse events that occurred throughout the study. Regular biweekly follow-ups were conducted by healthcare personnel via phone calls or in-person visits to inquire about potential side effects. No adverse events related to the intervention were reported.

Biomarker analysis

Blood samples were collected from participants at baseline (Day 0) and post-intervention (Day 84) following an 8-hour fasting period. Serum was separated by centrifugation (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany) and stored at -80°C until analysis. Levels of MMP-9 and NGAL were measured using commercially available ELISA kits from Abcam® (Cambridge, UK) according to the manufacturer's instructions. The MMP-9-NGAL ratio was calculated for each participant to assess changes in renal biomarkers. All samples were analyzed in batches within three months of collection to maintain biomarker stability, and uniform procedures were followed to minimize pre-analytical variability.

Glycemic control at baseline was assessed by measuring hemoglobin A1c (HbA1c) levels. Venous blood samples were collected after an overnight fast, and HbA1c was analyzed using HPLC (Bio-Rad D-10, USA). This parameter was used to characterize participants' glycemic status prior to intervention.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Between-group comparisons used independent t-tests for normally distributed data, Mann-Whitney U test for non-normal data, and Chi-square test for categorical variables. Within-group changes were analyzed with paired t-tests or Wilcoxon signed-rank tests. Effect sizes assessed intervention impact, with $p<0.05$ indicating significance. Analyses were performed using SPSS 26.0 (IBM Corp., Armonk, NY, USA). To account for the imbalance in educational level at baseline ($p=0.02$), sensitivity analyses were conducted using analysis of covariance (ANCOVA) models with educational level included as a covariate. These analyses aimed to rule out potential confounding effects of educational differences on the outcomes of MMP-9, NGAL, and the MMP-9-NGAL ratio. An interim analysis was conducted at week 6 to evaluate participant safety and adherence. No stopping rules were applied, and the study proceeded without modification. To control for type I error due to multiple testing across the three primary biomarkers (MMP-9, NGAL, and MMP-9-NGAL ratio), false discovery rate (FDR) correction was applied to the p-values.

Statistical analyses were conducted on a per-protocol basis, including only participants who completed the 12-week intervention and had complete data on both pre- and post-intervention biomarkers. All continuous data were tested for normality using the Shapiro-Wilk test. Biomarker data that deviated significantly from normal distribution were log-transformed prior to parametric testing to meet the assumptions of normality. For non-normally distributed data, non-parametric tests were applied.

RESULTS

Our findings demonstrate that the 12-week intervention with *P. crocatum*-enriched functional cookies

led to significant reductions in serum MMP-9, NGAL, and the MMP-9-NGAL ratio in patients with type 2 diabetes and early renal dysfunction. These results remained robust after adjusting for baseline educational level, indicating the potential therapeutic benefit of *P. crocatum* in mitigating inflammation-related biomarkers linked to diabetic kidney complications.

To assess the effectiveness of the intervention, baseline characteristics and biomarker levels were compared between the intervention and placebo groups. A balanced distribution of demographic and clinical variables ensures that observed effects can be attributed to the intervention rather than pre-existing differences. A total of 92.3% of participants in the intervention group demonstrated adherence above 80%, based on returned product counts during the 12-week intervention period. The reported findings are based on per-protocol analysis of 191 participants in the intervention group and 189 in the placebo group who completed the study with complete biomarker data.

Table 2 presents the baseline characteristics of participants in the intervention group (n = 191) and the placebo group (n = 189). There were no statistically significant differences between the two groups in terms of age, gender distribution, duration of DM, marital status, or the presence of other comorbidities. However, a significant difference was observed in the distribution of educational levels (p=0.02), with a higher percentage of participants holding a Bachelor/Diploma education in the intervention group and more participants with only an Elementary or Junior High School education in the placebo group. This difference in educational background should be taken into account when interpreting the results, as it may affect adherence to the intervention and health literacy. The mean baseline HbA1c level was 8.3% (±1.2) in the intervention group and 8.4% (±1.3) in the placebo group (p=0.53), indicating comparable glycemic control at the study's outset.

A 3-day dietary recall was conducted post-intervention in both groups to assess background dietary intake. The mean daily energy intake in the intervention group was 1,574 ± 219 kcal, with carbohydrate intake of 210.4 ± 35.2 g/day, protein intake of 57.3 ± 12.1 g/day, and fat intake of 53.9 ± 9.8 g/day. In the placebo group, mean energy intake was 1,592 ± 245 kcal, with carbohydrate, protein, and fat intakes of 216.7 ± 37.1 g/day, 56.8 ± 11.4 g/day, and 54.7 ± 10.3 g/day, respectively. No statistically significant differences were observed between the two groups in any of the macronutrient components (p>0.05), indicating comparable dietary patterns during the intervention period (Table 3).

Furthermore, the baseline levels of renal biomarkers (MMP-9, NGAL, and MMP-9-NGAL ratio) were comparable between the two groups, indicating that the initial clinical status was balanced before the intervention.

Table 4 illustrates the impact of intervention on biomarker levels and effect sizes among individuals diagnosed with DM. Regarding MMP-9 (ng/mL), both the intervention and placebo cohorts demonstrated notable alterations in mean levels before and after the intervention. In the intervention group, MMP-9 levels notably decreased from 518.45 ± 142.6 to 350.34 ± 134.15 ng/mL (p=0.0001), with a considerable mean change of -168.11 ± 97.67 ng/mL (95% CI: –185.2 to –151.0). Conversely, in the placebo group, the change was minimal (–7.95 ± 19.03 ng/mL, 95% CI: –10.6 to –5.3, p=0.014) (Table 3). The effect size, calculated as the mean change divided by the standard deviation, was markedly higher in the intervention group (-1.178) compared to the placebo group (-0.057), indicating a more pronounced intervention effect on MMP-9 levels. After applying FDR correction, the result remained statistically significant (FDR-adjusted p=0.0003), confirming the robustness of the finding.

Similarly, significant changes in NGAL levels (ng/mL) were observed pre- and post-intervention in both groups, as shown in Table 4. In the intervention group, NGAL levels significantly decreased from 195.67 ± 10.79 to 174.31 ± 17.90 ng/mL (p=0.0001), with a mean change of -21.35 ± 19.72 ng/mL (95% CI: –23.9 to –18.8). In contrast, the placebo group showed a non-significant change of –4.12 ± 28.65 ng/mL (95% CI: –6.9 to –1.3, p=0.453). The effect size was notably higher in the intervention group (-1.97), indicating a more pronounced intervention effect on NGAL levels compared to the placebo group. After applying the FDR correction, the observed difference remained statistically significant (FDR-adjusted p=0.0006), further confirming the robustness of the findings.

Furthermore, significant changes were observed in the MMP-9-NGAL ratio in both groups. In the intervention group, the ratio significantly decreased from 2.65 ± 0.73 to 1.99 ± 0.67 (p=0.0001), with a mean change of -0.66 ± 0.51 (95% CI: –0.72 to –0.60). Meanwhile, the placebo group experienced a negligible change of –0.01 ± 0.47 (95% CI: –0.06 to 0.04, p=0.867). The effect size was markedly higher in the intervention group (-0.904), indicating a more pronounced intervention effect on the MMP-9-NGAL ratio compared to the placebo group. After applying FDR correction, the result remained statistically significant (FDR-adjusted p=0.0011). Sensitivity analyses adjusting for educational level showed that the significant reductions in MMP-9, NGAL, and the MMP-9-NGAL ratio in the intervention group remained statistically significant (p<0.001). This indicates that educational level did not confound the relationship between the intervention

and biomarker outcomes. These trends for MMP-9, NGAL, and MMP-9-NGAL ratio levels are visually depicted in Fig. 2, highlighting the more pronounced effects in the intervention group compared to the placebo group.

Table 2. Participant characteristics in the Intervention and Placebo groups.

Variable	Intervention group (n = 191)	Placebo group (n = 189)	p-value
Age (years), mean ± SD	57.17 ± 0.56	55.85 ± 8.14	0.592[a]
Distance to healthcare facility (km), mean ± SD	3.05 ± 2.66	1.53 ± 0.89	0.317[b]
Duration of DM (years), mean ± SD	6.07 ± 3.47	3.99 ± 3.50	1.000[b]
Gender			1.000[c]
Male, n (%)	13.3% (25)	11.5% (22)	
Female, n (%)	86.7% (166)	88.5% (167)	
Educational Level			0.02[c]*
Elementary school, n (%)	20.0% (19)	65.4% (123)	
Junior high school, n (%)	23.3% (45)	0.0% (0)	
Senior high school, n (%)	26.7% (51)	15.4% (29)	
Bachelor/Diploma, n (%)	30.0% (76)	19.2% (37)	
Occupation			0.396[c]
Housewife, n (%)	73.3% (140)	76.9% (145)	
Retiree, n (%)	16.7% (32)	3.8% (7)	
Farmer, n (%)	3.3% (6)	7.7% (14)	
Civil-servant, n (%)	6.7% (10)	11.6% (23)	
Marital status			0.253[c]
Married, n (%)	90.0% (172)	81.8% (155)	
Widow/widower, n (%)	10.0% (19)	18.2% (34)	
HbA1c (%), mean ± SD	8.3 ± 1.2	8.4 ± 1.3	0.53[b]
MMP-9 pre (ng/mL)	518.45 ± 142.61	469.34 ± 140.01	0.184[a]
NGAL pre (ng/mL)	195.67 ± 10.79	188.38 ± 18.93	0.08[a]
MMP-9-NGAL ratio	2.65 ± 0.74	2.53 ± 0.85	0.567[a]

[a]Independent T-test; [b]Mann-Whitney U; [c]Chi-square; *significant. Values are presented as mean ± SD for continuous variables and as percentages for categorical variables.

Table 3. Comparison of mean daily nutrient intake based on 3-day dietary recall between Intervention and Placebo groups.

Nutrient Intake (per day)	Intervention (Mean ± SD)	Placebo (Mean ± SD)	p-value
Energy (kcal)	1,574 ± 219	1,592 ± 245	0.72
Carbohydrate (g)	210.4 ± 35.2	216.7 ± 37.1	0.48
Protein (g)	57.3 ± 12.1	56.8 ± 11.4	0.81
Fat (g)	53.9 ± 9.8	54.7 ± 10.3	0.67

Values are presented as mean ± standard deviation. Group comparisons were performed using independent t-tests.

Table 4. Intervention effectiveness on biomarker levels (MMP-9, NGAL, MMP-9-NGAL ratio) in patients with diabetes mellitus.

Variable	Group	Pre (mean ± SD)	Post[#] (mean ± SD)	p-value (Pre-Post)	Mean change ± SD	95% CI for mean change	p-value	Adjusted p-value (FDR)	Effect size (d=mean /SD)	95% CI for Cohen's [d]
MMP-9	Intervention	518.45 ± 142.6	350.34 ± 134.15	0.0001[a]*	-168.10 ± 97.67	−185.2 to −151.0	0.0001[c]*	0.0003*	-1.178	−1.46 to −0.90
(ng/mL)	Placebo	469.34 ± 140.01	461.38 ± 138.59	0.014 [a]*	-7.95 ± 19.03	−10.6 to −5.3		0.032	-0.057	−0.19 to 0.07
NGAL	Intervention	195.67 ± 10.79	174.31 ± 17.90	0.0001[b]*	-21.35 ± 19.72	−23.9 to −18.8	0.01[d]*	0.0016*	-1.97	−2.25 to −1.69
(ng/mL)	Placebo	188.38 ± 18.93	184.26 ± 20.57	0.453[b]	-4.12 ± 28.65	−6.9 to −1.3		0.482	-	−0.31 to 0.03
MMP-9-NGAL ratio	Intervention	2.65 ± 0.73	1.99 ± 0.67	0.0001[b]*	-0.66 ± 0.51	−0.72 to −0.60	0.0001[d]*	0.0011*	-0.904	−1.13 to −0.67
	Placebo	2.53 ± 0.85	2.51 ± 0.76	0.867[b]	-0.01 ± 0.47	−0.06 to +0.04		0.911	-	−0.15 to 0.11

Statistical tests: [a]Wilcoxon Signed-Rank Test, [b]Paired t-test, [c]Mann–Whitney U Test, [d]Independent t-test. Cohen's d effect sizes are accompanied by 95% confidence intervals. FDR: False discovery rate. *Significant results. [#]Post: 12 weeks.

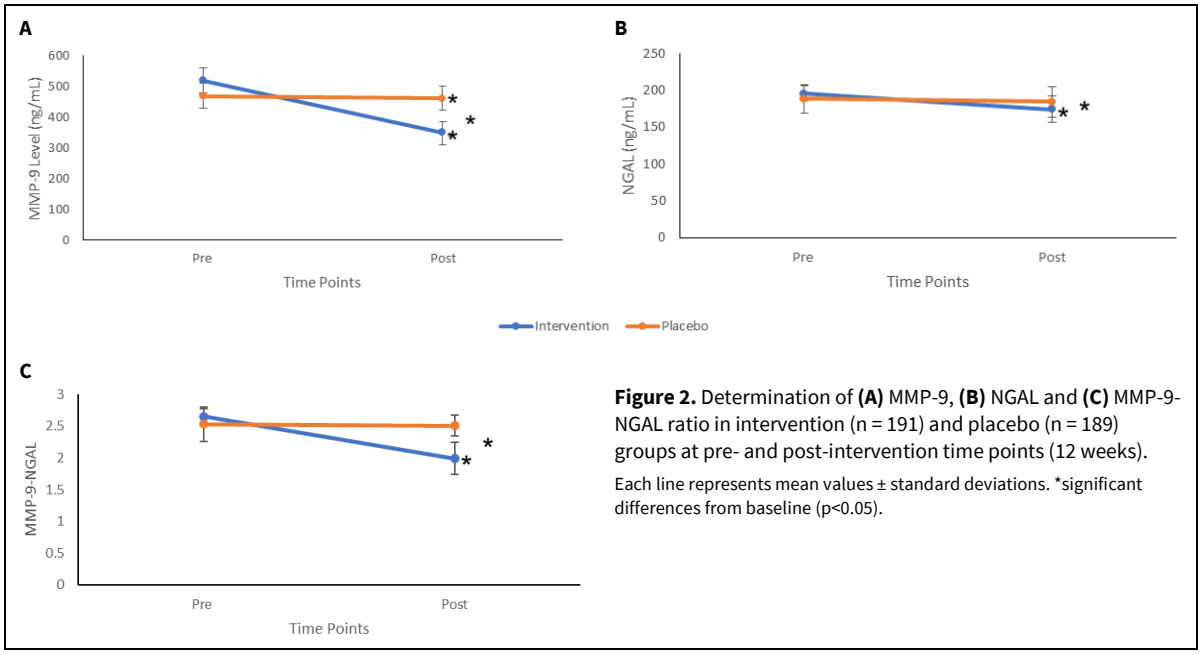

Figure 2. Determination of **(A)** MMP-9, **(B)** NGAL and **(C)** MMP-9-NGAL ratio in intervention (n = 191) and placebo (n = 189) groups at pre- and post-intervention time points (12 weeks).

Each line represents mean values ± standard deviations. *significant differences from baseline (p<0.05).

The effect sizes (Cohen's d) and their corresponding 95% CIs were calculated to quantify the magnitude and precision of the intervention effects. For MMP-9, the effect size in the intervention group was large (d = –1.18, 95% CI: –1.46 to –0.90), whereas in the placebo group, it was negligible (d = –0.06, 95% CI: –0.19 to 0.07). Similarly, for NGAL, the intervention group showed a large effect size (d = –1.97, 95% CI: –2.25 to –1.69), while the placebo group demonstrated a small and non-significant effect (d = –0.14, 95% CI: –0.31 to 0.03). The MMP-9-NGAL ratio also showed a substantial effect in the intervention group (d = –0.90, 95% CI: –1.13 to –0.67) compared to a trivial change in the placebo group (d = –0.02, 95% CI: –0.15 to 0.11).

As presented in Table 5, subgroup analysis demonstrated that the 12-week functional cookie intervention resulted in significant reductions in MMP-9 levels among several participant groups, including those aged <60 years (–158.3 ± 91.4 ng/mL; p=0.0003), females (–164.5 ± 95.2 ng/mL; p=0.0001), those with a diabetes duration ≥5 years (–178.9 ± 98.6 ng/mL; p=0.001), and individuals with poor glycemic control (HbA1c ≥8.0%) (–168.2 ± 96.1 ng/mL; p=0.0009). Significant reductions in NGAL and MMP-9-NGAL ratio were also observed in subgroups such as higher-educated participants (–22.9 ± 18.5 ng/mL; p=0.001 and –0.69 ± 0.45; p=0.003, respectively), civil servants, and those with HbA1c ≥8.0% (–20.7 ± 20.3 ng/mL; p=0.003 and –0.63 ± 0.50; p=0.002, respectively). Meanwhile, non-significant changes were found in older participants (age ≥60 years), those with elementary education, and farmers. These findings suggest that the functional food intervention had a more pronounced effect among individuals with higher disease burden, better education, and greater metabolic instability

DISCUSSION

This study demonstrated that *P. crocatum*-enriched functional foods significantly reduced serum MMP-9, NGAL, and their ratio in diabetic patients with early renal dysfunction. These reductions suggest attenuation of inflammation and oxidative stress, both of which are critical drivers of DN progression. The findings align with previous reports on the antioxidant and anti-inflammatory effects of *P. crocatum* polyphenols, which downregulate MMP-9 expression and mitigate

Table 5. Subgroup analysis of biomarker changes (MMP-9, NGAL, and MMP-9-NGAL ratio) following 12-week functional cookie intervention in patients with type 2 diabetes mellitus.

Subgroup	n	Mean change in MMP-9 ± SD	p-value MMP-9	Mean change in NGAL ± SD	p-value NGAL	Mean change in MMP-9-NGAL ratio ± SD	p-value MMP-9-NGAL ratio
Age <60 years	102	-158.3 ± 91.4	0.0003[a*]	-19.4 ± 18.2	0.008[a*]	-0.55 ± 0.42	0.011[a*]
Age ≥60 years	89	-110.1 ± 110.6	0.037[b*]	-8.5 ± 20.1	0.104[b]	-0.23 ± 0.56	0.087[b]
Duration of DM <5 years	85	-152.4 ± 90.2	0.018[a*]	-17.1 ± 18.7	0.029[a*]	-0.52 ± 0.49	0.033[a*]
Duration of DM ≥5 years	106	-178.9 ± 98.6	0.001[a*]	-23.0 ± 21.5	0.009[a*]	-0.64 ± 0.54	0.002[a*]
Male	47	-120.4 ± 92.5	0.009[b*]	-10.3 ± 22.1	0.077[b]	-0.31 ± 0.47	0.065[b]
Female	144	-164.5 ± 95.2	0.0001[a*]	-21.2 ± 19.3	0.002[a*]	-0.64 ± 0.49	0.005[a*]
Elementary education	64	-90.2 ± 115.0	0.138[b]	-6.1 ± 26	0.351[b]	-0.17 ± 0.51	0.192[b]
Higher education	127	-172.8 ± 89.3	0.0001[a*]	-22.9 ± 18.5	0.001[a*]	-0.69 ± 0.45	0.003[a*]
Housewife	140	-163.0 ± 94.5	0.003[a*]	-18.4 ± 18.6	0.067[a]	-0.50 ± 0.51	0.059[a]
Retiree	32	-175.6 ± 97.3	0.002[b*]	-21.9 ± 21.2	0.018[b*]	-0.58 ± 0.52	0.005[b*]
Farmer	9	-159.4 ± 92.0	0.047[b*]	-17.7 ± 16.4	0.107[b]	-0.41 ± 0.47	0.118[b]
Civil-servant	10	-185.2 ± 99.5	0.001[b*]	-23.7 ± 21.7	0.005[b*]	-0.70 ± 0.57	0.001[b*]
HbA1c ≥ 8.0%	78	-168.2 ± 96.1	0.0009[a*]	-20.7 ± 20.3	0.003[a*]	-0.63 ± 0.50	0.002[a*]

Values are presented as mean change ± standard deviation. Statistical tests used: [a]Paired t-test; [b]Wilcoxon signed-rank test. A *p-value <0.05 was considered statistically significant.

tubular injury biomarkers (Papadopoulou-Marketou et al., 2021).

Biomarkers such as MMP-9 and NGAL are essential in assessing renal inflammation and oxidative stress in DN, where ECM remodeling and tubular injury play significant roles in disease progression (Liu et al., 2021). MMP-9 is a proteolytic enzyme involved in ECM degradation, and its overexpression has been linked to the breakdown of glomerular integrity, contributing to kidney fibrosis and chronic inflammation (Garcia-Fernandez et al., 2020). The upregulation of MMP-9 in DN reflects ongoing inflammatory processes that exacerbate renal injury, highlighting its potential as a marker for disease progression and severity in diabetic patients (Cione et al., 2020; Russa et al., 2024).

NGAL, on the other hand, is a sensitive marker for tubular damage and an early indicator of renal dysfunction. Elevated NGAL levels correlate with acute and chronic kidney injuries, particularly in DN, where oxidative stress and metabolic dysregulation lead to tubular cell apoptosis and impaired filtration function (Megallaa et al., 2023). As DN advances, the increase in NGAL signifies a higher risk of progression to ESRD (Danquah et al., 2023). The MMP-9-NGAL ratio, therefore, provides a composite view of both glomerular and tubular health, making it a valuable biomarker for evaluating the balance between ECM remodeling and tubular integrity in DN. This ratio serves as a potential marker not only for disease severity but also for monitoring treatment responses, as therapies that reduce MMP-9 or NGAL levels may indicate decreased renal inflammation and improved renal function (Romejko et al., 2023).

The observed reduction in MMP-9 in this study highlights *P. crocatum*'s beneficial impact on ECM remodeling, a process often dysregulated in DN due to heightened proteolytic activity (Cabral-Pacheco et al., 2020). MMP-9 overexpression is closely linked to renal tissue damage and fibrosis, exacerbating the progression of nephropathy by degrading ECM components essential for structural stability (Rodríguez-Sánchez et al., 2020). By lowering MMP-9 levels, *P. crocatum* may help preserve ECM integrity, mitigate the risk of fibrosis, and potentially slow down DN progression. This outcome aligns with previous research demonstrating the antioxidant and anti-inflammatory properties of *P. crocatum*, primarily attributed to its rich content of flavonoids and glycoside polyphenols, which are known to downregulate MMP-9 expression in inflammatory conditions (Bae et al., 2017; Li et al., 2019).

In addition to MMP-9, the significant reduction in NGAL levels in the intervention group underscores *P. crocatum*'s potential to protect against tubular injury. NGAL, an early and sensitive biomarker of renal tubular damage, is frequently elevated in DN due to oxidative stress-induced damage in renal tubular cells

(Kovacevic et al., 2021). The antioxidative properties of *P. crocatum* appear to protect these cells, as evidenced by the observed decrease in NGAL levels, suggesting a reduction in oxidative stress on tubular cells (Nevo et al., 2018). This aligns with studies indicating that polyphenolic compounds from *P. crocatum* can protect against cellular oxidative damage, further supporting renal health (Ashkar et al., 2022).

The observed modulation of MMP-9 and NGAL levels suggests that *P. crocatum* may influence oxidative stress and inflammatory pathways. Although previous studies indicate interactions with proteins such as SOD1, p53, and E-cadherin, direct mechanistic validation in this trial was not performed and warrants further investigation (Addo-Preko et al., 2023). By upregulating SOD1, a critical enzyme for neutralizing reactive oxygen species (ROS), *P. crocatum* reduces oxidative stress, thereby lowering both MMP-9 and NGAL expression (Ayuk et al., 2016). Reduced ROS levels prevent the activation of pathways that would otherwise lead to MMP-9-driven ECM degradation and NGAL-mediated tubular injury, thus maintaining the stability of renal structures.

Moreover, *P. crocatum* modulates p53, a pro-apoptotic protein that, when elevated in DN, can exacerbate tissue damage and promote MMP-9 activity as part of the remodeling response (Pan et al., 2018). By downregulating p53, *P. crocatum* reduces apoptosis, preserving cell viability and minimizing unnecessary ECM breakdown (Hernández Borrero and El-Deiry, 2021). This moderation of p53 also indirectly protects tubular cells from stress-induced damage, thereby contributing to the reduction of NGAL. Furthermore, *P. crocatum* upregulates E-cadherin, a cell adhesion protein crucial for tissue cohesion and stability. High levels of MMP-9 often correlate with reduced E-cadherin, leading to increased cell detachment and structural disorganization (Rojas-Quintero et al., 2018). By promoting E-cadherin expression, *P. crocatum* counteracts MMP-9's detrimental effects, supporting renal epithelial integrity and preventing tubular disarray (Zakiyanov et al., 2019).

Collectively, these findings suggest that *P. crocatum* exerts a multi-targeted protective mechanism in DN, simultaneously modulating glomerular and tubular health through regulation of MMP-9, NGAL, SOD1, p53, and E-cadherin. These effects suggest *P. crocatum* is a promising adjunctive therapy for DN. It may reduce inflammation and oxidative stress, while preserving ECM and tubular integrity.

The MMP-9-NGAL ratio, which provides a composite measure of glomerular and tubular health, also decreased significantly in the intervention group. This reduction suggests that *P. crocatum* impacts multiple pathways relevant to DN pathophysiology, making it a promising adjunct therapy for managing renal inflammation and oxidative stress in diabetes. The modulation of both glomerular (MMP-9) and tubular (NGAL) markers supports the hypothesis that *P. crocatum* can simultaneously influence multiple facets of renal health, potentially providing a more comprehensive protective effect.

Notably, individuals with poor glycemic control (HbA1c ≥ 8.0%) showed significant reductions in MMP-9, NGAL, and the MMP-9-NGAL ratio following the 12-week intervention. This highlights the potential of *P. crocatum*-enriched functional foods to provide greater benefit among patients with uncontrolled diabetes, likely due to the heightened baseline inflammatory and oxidative burden in this group (Mallik et al., 2024). Similarly, participants who were younger, female, had a longer duration of diabetes, or possessed higher educational backgrounds tended to show more favorable reductions in inflammatory and oxidative stress markers (Wong et al., 2024; Xie et al., 2020). These differences may reflect varying levels of health literacy, adherence, or physiological responsiveness to dietary interventions (Siopsis et al., 2021). Notably, individuals with greater educational attainment may have been more receptive to the behavioral components of the intervention, enhancing its overall impact (Cusack et al., 2018). Likewise, gender-related metabolic or hormonal differences could contribute to varying degrees of biomarker modulation (Costanzo et al., 2022). Although some occupational groups exhibited less pronounced changes, the overall trend across subgroups supported the consistency of the intervention's effect. These findings underscore the significance of participant characteristics in influencing therapeutic outcomes and highlight the importance of personalized approaches in nutritional interventions for diabetes management.

Our findings are consistent with broader phytotherapeutic research, which indicates the efficacy of plant-based compounds in attenuating renal damage. For instance, recent studies have demonstrated that phytochemicals such as asiaticoside from *Centella asiatica* and berberine from *Berberis aristata* can suppress NF-κB activation and reduce oxidative stress, both of which play central roles in the pathogenesis of DN (Belwal et al., 2020; Legiawati et al., 2018). These mechanistic parallels suggest that *P. crocatum* may exert its renal benefits through similar pathways, although further experimental validation is warranted.

Despite the promising results, this study has some limitations. However, blinding was maintained throughout the trial, and a post-trial blinding assessment suggested that participants were generally unaware of their group allocation, reducing the likelihood

of bias related to treatment expectancy. In addition to MMP-9 and NGAL, conventional renal parameters, including serum urea, serum creatinine, estimated glomerular filtration rate (eGFR), and urine albumin, were also measured. The intervention group showed favorable trends, including a modest increase in eGFR and a slight reduction in urine albumin. However, these changes were not statistically significant during the 12-week period. These results align with previous reports that classical renal function markers may not be sufficiently sensitive to detect early subclinical improvements in renal function in short-duration interventions (Danijela et al., 2016; Quaglia et al., 2020). The analysis focused on MMP-9 and NGAL, as these biomarkers more sensitively captured early inflammatory and oxidative responses. Although conventional renal parameters were excluded from the main results, their observed trends aligned with potential renal improvement and merit further evaluation in future studies with extended follow-up periods of 6 to 12 months. Additionally, further mechanistic studies are required to elucidate the precise pathways through which *P. crocatum* regulates MMP-9 and NGAL. Importantly, the observed effects of the intervention remained robust after adjusting for educational level in sensitivity analyses. This suggests that the beneficial changes in biomarker levels were not confounded by differences in health literacy or adherence, potentially linked to educational background, further strengthening the validity of our findings. Additionally, to strengthen the validity of the findings, FDR correction was used to adjust for multiple comparisons involving the primary biomarkers. Additionally, a planned interim analysis at week 6 confirmed no adverse events or deviations in compliance, supporting the continued safety and integrity of the intervention protocol.

Future research should include mechanistic analyses such as qPCR and Western blot to evaluate the expression of MMP-9, NGAL, SOD1, and p53, supported by in vitro studies using renal tubular cells. Expanding the biomarker panel, such as malondialdehyde (MDA), glutathione, eGFR, kidney injury molecule-1 (KIM-1), and cystatin-C, may offer deeper insight into the nephroprotective mechanisms and better capture the subclinical progression of DN. Although a 3-day dietary recall was conducted post-intervention, prospective dietary monitoring is recommended to minimize potential confounding factors. The absence of objective biomarkers of compliance, such as plasma or urinary polyphenol levels, limits the ability to biochemically verify participant adherence. Future studies should incorporate such measures to strengthen intake validation. Lastly, dose–response trials and extended follow-up (≥6 months) are needed to assess long-term and cumulative effects. To support the feasibility of the intervention, a separate organoleptic study involving 50 panelists demonstrated favorable sensory acceptance of the cookies, encompassing taste, aroma, and texture. These findings, reported in a manuscript currently in press, suggest that the product is well tolerated and palatable, which is essential for long-term adherence in nutritional interventions (Setyawati et al., 2025).

CONCLUSION

This randomized trial offers preliminary evidence that *P. crocatum*-based functional foods may serve as a promising adjunctive therapy for DN by modulating key renal biomarkers. The intervention significantly reduced serum MMP-9, NGAL, and the MMP-9-NGAL ratio in patients with type 2 diabetes and early renal dysfunction, suggesting nephroprotective effects through attenuation of inflammation and oxidative stress. These findings align with the therapeutic potential of *P. crocatum* in addressing the early pathophysiology of diabetic kidney disease. Although the product demonstrated favorable acceptability and biological effects, the relatively short intervention period and absence of in-depth mechanistic validation limit the generalizability of the results. Future research with extended follow-up and molecular analyses is warranted to confirm the durability of the observed effects and elucidate the underlying pathways.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. Due to privacy and ethical restrictions, individual-level data cannot be shared publicly. Aggregated data used to support the findings of this study can be made available upon request for research and verification purposes.

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AUTHOR CONTRIBUTION:

Contribution	Setyawati A	Jafar N	Raihan M	Kristianto H
Concepts or ideas	x			
Design	x		x	
Definition of intellectual content		x		x
Literature search	x	x	x	x
Clinical trial	x	x		
Data acquisition	x	x	x	x
Data analysis	x	x	x	x
Statistical analysis	x			
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

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