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Original Article

Protective effect of yacon leaves extract (*Smallanthus sonchifolius* (Poepp.) H. Rob) through antifibrosis, anti-inflammatory, and antioxidant mechanisms toward diabetic nephropathy

Wahyu Widowati[1,*], Rita Tjokropranoto[1], Philips Onggowidjaja[1], Hanna Sari Widya Kusuma[2], Cahyaning Riski Wijayanti[2], Meganita Marthania[2], Afif Yati[2], and Rizal Rizal[2,3]

[1]Faculty of Medicine, Maranatha Christian University, Jl. Surya Sumantri No. 65, Bandung 40164, West Java, Indonesia.
[2]Biomolecular and Biomedical Research Center, Aretha Medika Utama, Jl Babakan Jeruk II No. 9, Bandung 40163, West Java, Indonesia.
[3]Biomedical Engineering, Department of Electrical Engineering, Faculty of Engineering, Universitas Indonesia, Depok 16426, West Java, Indonesia.

Abstract

Background and purpose: Diabetic nephropathy (DN) is a chronic kidney failure, which may lead to fatality. Mesangial cell proliferation, renal inflammation, stress oxidative, and fibrosis are involved in DN progression. Yacon leaves (*Smallanthus sonchifolius* (Poepp.) H. Rob.) contains large amounts of phenolic compounds and it has the ability to inhibit oxidative stress, inflammation, and fibrosis. Considering the potential of yacon leaves extract (YLE), it may be used for DN treatment. This research aimed to elucidate YLE's potential as anti-DN through anti-inflammatory, antioxidant, and antifibrosis mechanisms.
Experimental approach: Mesangial cells were induced by glucose 20 mM for 5 days and treated with YLE concentrations as much as 5, 10, and 50 µg/mL. TGF-β1, TNF-α, and MDA levels were measured using the ELISA method. SMAD2, SMAD3, SMAD4, and SMAD7 gene expressions were analyzed using the qRT-PCR method.
Findings/Results: YLE at 5, 10, and 50 µg/mL could reduce the levels of TGF-β1, TNF-α, and MDA compared with the DN cells model. YLE could reduce gene expressions of SMAD2, SMAD3, and SMAD4 and increase SMAD7 expression.
Conclusion and implications: YLE potentially mitigated diabetic nephropathy through antifibrosis, anti-inflammatory, and antioxidant capacities.

Keywords: Diabetic nephropathy; MDA; SMADs; TGF-β1; TNF-α; Yacon leaves extract.

INTRODUCTION

Diabetic nephropathy (DN) is generated by chronic kidney failures, leads to chronic kidney disease (CKD) and end-stage renal disease, then ultimately causes fatality (1,2). Glomeruli damage causes protein loss to the urine, consequently increasing serum albumin, thereby generating body swelling in nephrotic syndrome (3). Furthermore, mesangial cells and the extracellular matrix invade nephrons and interrupt the filtration process (4). Therefore, its severity and complicated mechanisms still need to be explained profoundly in order to develop advanced therapy. The excessive extracellular matrix protein in the mesangial interstitial may cause glomerulosclerosis, which results in fibrosis that can take the form of diffuse or nodular alterations (5). A master regulator cytokine that mediates these effects has been identified as Transforming Growth Factor β-1 (TGF-β1) (6). All types of cells within the kidney are able to synthesize TGF-β1 (7).

*Corresponding author: W. Widowati
Tel & Fax: +62-62222017621
Email: wahyu.widowati@maranatha.edu

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The TGF-β1 level is strongly excessive in proximal tubule damage, and its activity reflects the severity of the injury (7). Some studies show that severe renal disease could cause peritubular capillary loss and microvascular rarefaction associated with increasing TGF-β1 signaling (8). The major intracellular signaling mechanism which transduces TGF-β1 is Small Mothers Against the Decapentaplegic (SMAD) pathway (9). Renal fibrosis may be mediated by the activation of SMAD2 and SMAD3 (10). A variety of roles for SMAD4 are involved in kidney fibrosis and inflammation. In contrast, SMAD7 plays a protective role against kidney fibrosis and inflammation by inhibiting SMAD2 and SMAD3 activations (11).

DN is also signified by the increase in inflammation levels. This condition is associated with the increase of interstitial extracellular matrix components, profibrotic growth factors, and pro-inflammation cytokines (5). Tumor Necrosis Factor-α (TNF-α) is a mediator of cytokines for both chronic and acute inflammation (12). It also indicates severe DN (13). The main triggers that cause inflammation brought on by hyperglycemia are what cause kidney damage and nephropathy (14). By altering the quantity of antioxidant enzymes in kidney cells and producing too many Reactive Oxygen Species (ROS), hyperglycemia results in the induction of oxidative stress (15). Malondialdehyde (MDA) level could be measured to evaluate the levels of free radicals and oxidative stress (16).

Yacon (*Smallanthus sonchifolius* (Poepp.) H. Rob) is a native plant of Andean that is widely consumed in South America. Yacon leaves extract (YLE) is reported to contain large amounts of phenolic compounds, mainly protocatechuics, chlorogenic, ferulic, gallic, rosmarinic, caffeic, and gentisic acids (17). Its role as an antioxidant has also been evinced (18). However, the fibrosis inhibition mechanism involving SMAD7 and the exact effective concentration of ethanolic YLE has not been declared. Considering the potential of YLE for novel DN treatment, this study has the objective to explain the anti-inflammatory, antioxidant, and anti-fibrosis potentials of certain concentrations of ethanolic YLE on DN cells models by measuring the levels of MDA, TGF-β1, and TNF-α proteins; and SMAD2, SMAD3, SMAD4, and SMAD7 gene expressions.

MATERIALS AND METHODS

YLE preparation

The yacon leaves were obtained from Bandung, Indonesia, and determined by Mr. Djuandi, Biology Department, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The sample number was 0220419-A008 and the determination confirmed that it was *Smallanthus sonchifolius* (Poepp.) H. Rob.

There was 2000 g of yacon leaves dried using a food dehydrator (LocknLock, EJO316 South Korea) at 37 ℃ for 3 d, then it was ground and submersed in 70% ethanol for 72 h. Every 24 h, the filtrate was collected and maceration was repeated 3 times. The filtrate was filtered using Whatman filter paper. The latest filtrate was concentrated using a rotary evaporator at 50 ℃ (Zhengzhou Well-known, RE-201D, China) for obtaining YLE, and then it was stored at -20 °C (19,20).

Preparation of DN cells model

SV40 MES 13 *Mus musculus* mesangial cell line (ATCC® CRL-1927™) was obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, West Java, Indonesia. The SV40 MES 13 cells (1×10^5) were plated in 6 well plates (Corning Costar, 3516, Germany). The medium was formulated with Dulbecco's modified eagle medium (DMEM; Biowest, L0103-500, France) and F12-K mix nutrient (Biowest, L0135-500, France) with comparison 1 : 3, 5% of fetal bovine serum (FBS; Biowest, S1810-500, France), 0.1% gentamicin (Gibco, 15750060, USA), 1% antibiotic-antimycotic (Biowest, L0010-100, France). The cell culture was incubated at 37 °C, and 5% CO_2 for 24 h (21). Afterward, the medium was replaced with 1800 µL of 20 mM glucose-induced medium (22) and added with 200 µL of YLE (at final concentrations 5, 10, 50 µg/mL) (23-25). Cells were incubated at 37 °C with 5% CO_2 for 5 days (24-25). Glucose-induced cells were defined as the positive control (PC), and

normal cells were defined as the negative control (NC).

Total protein assay

To assess the quantity of TGF-β1, TNF-α, and MDA in mg/protein, the total protein was quantified. Bovine standard albumin was used for this test (Sigma Aldrich, A9576, USA), and 1000 µL of ddH_2O containing 2 mg was used as stock solutions. Bovine standard albumin at different concentrations and 200 mL of quick start dye reagent 1X (Bio-Rad, 5000205, USA) were added to the well along with 20 mL of sample. The plate was incubated for 5 min at room temperature and the samples' absorbance was detected at 595 nm (Multiskan GO Microplate spectrophotometer, Thermo Fisher Scientific, USA) (26,27).

TGF-β1, TNF-α, and MDA assays

TGF-β1, TNF-α, and MDA levels of the conditioned medium or protein secretion of the samples were determined by ELISA assay using the mouse TGF-β1 (Elabscience, E-EL-M0051, China), TNF-α (Elabscience, E-EL-M0049, China), and MDA (Elabscience, E-EL-0060, China) ELISA kits following manufacturer's protocols, then the absorbance were all read at 450 nm using microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, USA) (24-26).

SMADs genes expressions measurements

RNA was extracted from the collection using RNA isolation kit (Zymo, R2073, USA), then continued with cDNA synthesis using IScript Reverse Transcription Supermix (Bio-Rad, 170-8841, USA) for real-time polymerase chain reaction (qRT-PCR) in accordance with the manufacturer's procedures. SsoFast EvaGreen Supermix was used in qRT-PCR following the manufacturer's instructions to assess the gene expression (Bio-Rad, 172-5200, USA), then run in AriaMx RT-PCR (Agilent, MY202205294, USA). Using the Cq approach, the expression of the genes was quantified as a relative copy number compared to the control (26). In Table 1, the primer sequences for SMAD2, SMAD3, SMAD4, SMAD7, and GAPDH are displayed. In Table 2, the RNA concentration and purity are displayed.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) 16.0 program was used to conduct the statistical analysis. The results were analyzed using one-way analysis of variance (ANOVA), then followed by Tukey post hoc test, and Dunnet-T3 post hoc test. $P \leq 0.05$ was taken into account to denote a significant difference for all treatments.

Table 1. Primers' sequences.

Gene	Primer sequence (5'-3') Upper strand: sense Lower strand: antisense	Product size (bp)	Annealing (°C)	Cycle	Reference
GAPDH	TCAAGATGGTGAAGCAG ATGTAGGCCATGAGGTCCAC	217	59	40	NCBI Reference sequence: NM_001289726
SMAD2 Mouse	ATTACATCCCAGAAACACCAC TAGTATGCGATTGAACACCAG	196	59	40	NCBI Reference sequence: NM_001252481.1
SMAD3 Mouse	GTAGAGACGCCAGTTCTACCT CATCTTCACTCAGGTAGCCAG	178	59	40	NCBI Reference sequence: NM_016769.4
SMAD4 Mouse	GAGAACATTGGATGGACGAC ACATACTTGGAGCATTACTCTG	242	54	40	NCBI Reference sequence: NM_001364967.1
SMAD7 Mouse	ACTCTGTGAACTAGAGTCTCCC CTCTTGGACACAGTAGAGCCT	241	59	40	NCBI Reference sequence: NM_001042660.1

Table 2. RNA Concentration and purity.

Sample	RNA Concentration (ng/µL)	RNA Purity (260/280 nm)
Normal cells	43.37	2.0776
Glucose-induced mesangial cells	60.16	2.2706
Glucose-induced cells treated with YLE at 5 µg/mL	64.20	2.2518
Glucose-induced cells treated with YLE at 10 µg/mL	47.72	2.4946
Glucose-induced cells treated with YLE at 50 µg/mL	21.84	1.6521

RESULTS

YLE preparation

This study began with processing fresh yacon leaves to be an extract. The fresh leaves were dried, then extracted, and finally resulted in a 15.78% extract yield. The details of the weights during the process are presented in Table 3.

Mesangial cells morphology as glucose induction and YLE treatments

In this study, CKD cells were generated by 20 mM glucose induction for 24 h. The effects of glucose induction and YLE toward mesangial cells morphology can be seen in Fig. 1. Mesangial excessive proliferation was observed and defined as the indication of DN (Fig. 1B). YLE treatments applied in this study inhibited the proliferation as the indication of DN alleviation (Fig. 1C-E). Proliferation inhibition increased as the rise of concentrations, therefore YLE at 50 µg/mL generated the highest inhibition.

The levels of TGF-β1, TNF-α, and MDA

The effect of YLE on TGF-β1, TNF-α, and MDA levels of the DN cells model are shown in Fig. 2.

Table 3. The yield of yacon leaf extract.

Wet leaves weight (g)	Dry weight of leaves (g)	Extract weight (g)	Extract yield (%)
2000	300	47.35	15.78

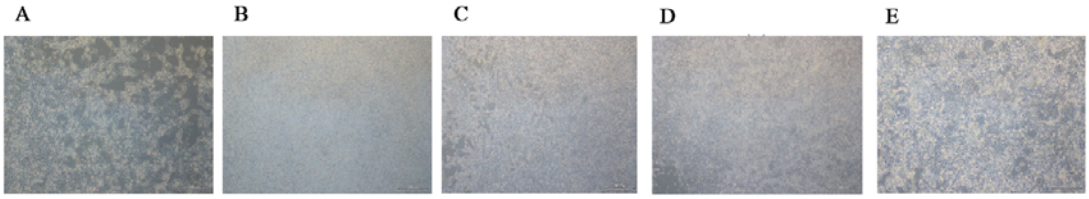

Fig. 1. Effect of YLE toward cell morphology on diabetic nephropathy cells model. (A) normal cells, (B) glucose-induced cells, (C) glucose-induced cells treated with YLE at 5 µg/mL, (D) glucose-induced cells treated with YLE at 10 µg/mL, and (E) glucose-induced cells treated with YLE at 50 µg/mL. YLE, Yacon leaves extract.

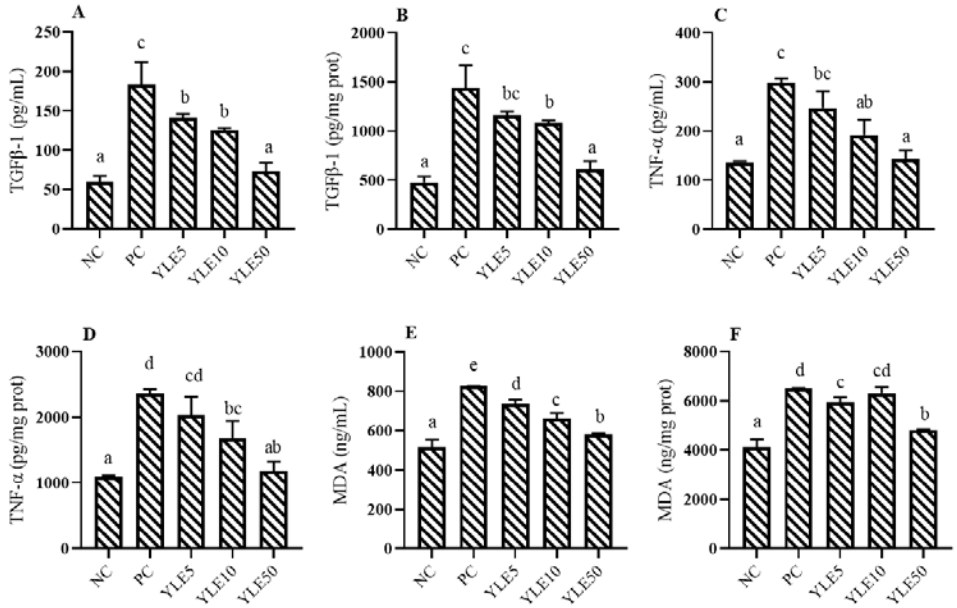

Fig. 2. The effect of YLE against the levels of (A-B) TGF-β1, (C-D) TNF-α, and (E-F) MDA on glucose-induced mesangial cells. The presented data are mean ± SD. YLE, Yacon leaves extract; TGF, transforming growth factor; TNF, tumor necrosis factor; MDA, malondialdehyde; NC, normal cells; PC, positive control which is mesangial cells induced by glucose; YLE 5, glucose-induced cells treated with YLE at 5 µg/mL; YLE 10, glucose-induced cells treated with YLE at 10 µg/mL; YLE 50, glucose-induced cells treated with YLE at 50 µg/mL. Different letters on the columns indicate significant differences ($P \leq 0.05$) among groups. Similar letters on the columns indicate no statistical differences ($P > 0.05$) among groups.

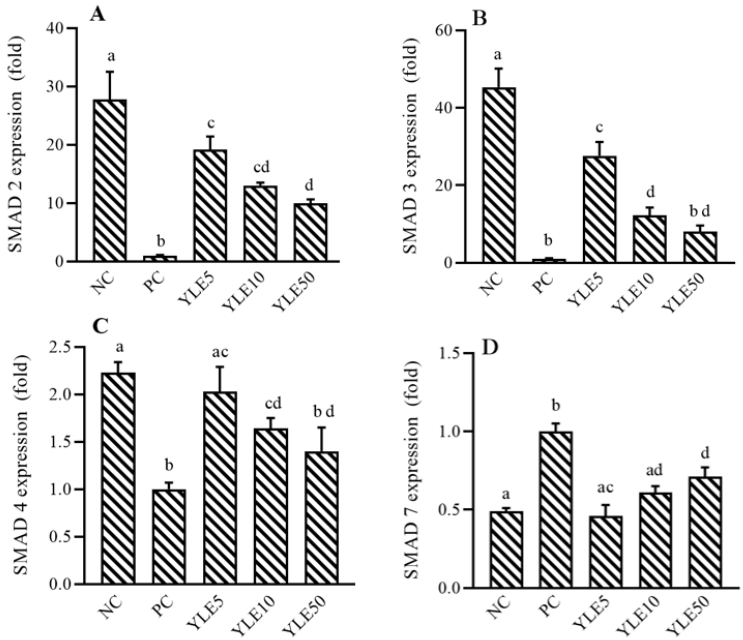

Fig. 3. (A-D) Relative SMAD2, SMAD3, SMAD4, and SMAD7 gene expressions on glucose-induced mesangial cells. The presented data are mean value ± SD. YLE, Yacon leaves extract; SMAD, small mothers against decapentaplegic; NC, normal cells; PC, positive control which is mesangial cells induced by glucose; YLE 5, glucose-induced cells treated with YLE at 5 µg/mL; YLE 10, glucose-induced cells treated with YLE at 10 µg/mL; YLE 50, glucose-induced cells treated with YLE at 50 µg/mL. Different letters on the columns indicate significant differences ($P \leq 0.05$) among groups. Similar letters on the columns indicate no statistical differences ($P > 0.05$) among groups.

Glucose treatment (20 mM) significantly increased TGF-β1, TNF-α, and MDA levels compared to untreated cells. The mentioned protein levels subsequently decreased following YLE treatment. YLE at 50 µg/mL was the most active in reducing TGF-β1, TNF-α, and MDA levels.

SMADs genes expressions

The effects of YLE on SMAD2, SMAD3, SMAD4, and SMAD7 gene expressions are shown in Fig. 3. The results showed that glucose-induced increasing SMAD2, SMAD3, and SMAD4 compared to normal cells. YLE significantly decreased the SMAD2, SMAD3, and SMAD4 expressions, and YLE at 50 µg/mL was the most active treatment to lower SMAD2, SMAD3, and SMAD4 gene expressions. Glucose significantly downregulated the SMAD7 expression compared to untreated cells; while YLE at 10 and 50 µg/mL significantly upregulated the SMAD7 expression compared to the positive control (diabetic nephropathy model).

DISCUSSION

DN is acknowledged as the main cause of the end-stage renal disease (28). The pathogenesis is indicated by mesangial proliferation in the nephron (4) and involves various pathways, including mitochondrial damage and ROS, and renal fibrosis. These two mentioned pathways are interrelated. ROS leads to cellular damage which then causes renal fibrosis (3,24-26,29).

YLE is considered to have a therapeutic effect on diabetes due to its bioactive compounds. It has polyphenolic compounds, including chlorogenic acid, *p*-coumaric acid, quercetin, protocatechuic acid, and ferulic acid. Furthermore, quercetin is confirmed as a major bioactive compound of ethanolic YLE (26,28). These compounds are known for their antioxidant and anti-inflammatory properties. On the other hand, ethanolic YLE is also known to contain sesquiterpene lactones, which have a role in nitric oxide formation to trigger inflammation and vasodilation reactions (19).

The cell morphology showed that glucose at 20 mM induced high cell proliferation. YLE potentially inhibits cell proliferation, which was indicated by lower cells density compared to the glucose-induced cells as DN cells model (Fig. 1). A previous study reported that naringin, one of YLE flavonoids, inhibited glucose-induced mesangial cell proliferation (30). YLE flavonoid namely quercetin inhibited glucose-induced mesangial cell proliferation, the DN cells model (26). In this study, higher YLE concentration caused higher proliferation inhibition. It is clearly observed that YLE 10 at µg/mL (Fig. 1D) inhibition is more effective than YLE at 5 µg/mL (Fig. 1C), while the best treatment was YLE at 50 µg/mL (Fig. 1E).

In the present study, excessive ROS level was assessed through MDA level. MDA can be used as a marker to examine oxidative stress and free radical damage (31), associated with DN in both clinical situations and biomedical research (17). This study demonstrated that the level of MDA of glucose-induced cells in the DN cells model was the highest among the groups. As the results of YLE treatments (5 to 50 µg/mL), the MDA level was significantly lowered. These results confirmed a previous study that ethanolic YLE potentially reduced ROS regarding free radical scavenging of 2,2-diphenyl l-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), hydrogen peroxide (H_2O_2), and ferric reducing antioxidant power (FRAP) assay (20,32). Moreover, this study showed a novel finding pertaining to the reduction of MDA levels by testing various concentrations of YLE. At 50 µg/mL concentration of YLE treatment, the MDA level of the glucose-induced cells was almost equivalent to the level of MDA of normal cells. It means that YLE treatment at 50 µg/mL could reduce the MDA level in DN condition.

The following elucidated mechanism is renal inflammation which is initiated by the abundant presence of macrophage, then it secretes inflammatory cytokines, including TNF-α and TGF-β1 (19,26). TNF-α is naturally produced by the activities of macrophages and monocytes on normal and malignant cells. Recent studies reported that TNF-α levels as inflammatory biomarkers increased significantly among patients with DN compared to controls without DN (33). Severe inflammation is a factor that is highly associated with DN. TNF-α is a mediator of cytokines in chronic and acute inflammations (13). Based on the results, the level of TNF-α on the glucose-induced cells as DN cells model (PC) was the highest level compared to the YLE treatment group. According to the results, the treatments of YLE (5 to 50 µg/mL) were significantly able to reduce the level of TNF-α produced by the DN cells model. At 50 µg/mL concentration of YLE, the TNF-α level was almost equivalent to the TNF-α level on the untreated (normal) cells. Moreover, the TNF-α level of YLE 50 µg/mL-treated cells was not significantly different from the normal cells. It implies that the treatment of YLE at that concentration could reduce the TNF-α level in DN condition and recover to normal, which corresponds to the previous research in which quercetin, one of the YLE flavonoids, could reduce TNF-α level on CKD cells model (26).

TGF-β1 is a pleiotropic inflammatory cytokine that is strongly exceeded in the injured proximal tubule, and the activity of TGF-β1 reflects the severity of the injury. Some studies show that severe renal disease could cause peritubular capillary loss and microvascular rarefaction associated with increased TGF-β1 signalling (8). The results of this study showed that the level of TGF-β1 of glucose-induced cells was the highest among the groups. Based on the results, the treatments of YLE (5 to 50 µg/mL) were able to significantly reduce the level of TGF-β1 produced by the DN cells model. This result was in line with the previous study in which ethanolic YLE could inhibit TGF-β1 expression and decrease fibrosis (28). In the YLE of 50 µg/mL, TGF-β1 level was comparable with the TGF-β1 level in untreated (normal) cells. It implies that the treatment of YLE with that concentration could possibly reduce the TGF-β1 level in DN condition and recover to normal condition.

The TGF-β superfamily of receptors includes receptors known as SMADs, which

are the family of structural proteins that served as the primary signal transducers for these receptors. There are three different sub-types of SMADs: inhibitory SMADs, common partner SMADs, and receptor-regulated SMADs (R-SMADs). Each of these three groups contains one of the eight SMADS family members. The R-SMADs are practiced in direct signaling from the TGF-β receptor and are made up of SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8/9. The sole known human Co-SMAD and SMAD4 collaborates with R-SMADs to enlist co-regulators for the complex (34). Finally, I-SMADs, including SMAD6 and SMAD7, have a function to inhibit R-SMADS activity (35). SMAD6 is more closely associated with bone morphogenetic protein signaling than SMAD7, which is a broad TGF-β signal inhibitor. R/Co-SMADs are mainly found in the cytoplasm, but after TGF-β signaling, they build up in the nucleus, the location for binding with DNA and transcription control. I-SMADs, however, are mostly located in the nucleus, where they have the potential to function as direct transcriptional regulators (36). According to the findings, glucose-induced cells had the highest levels of SMAD2, SMAD3, and SMAD4 compared to other treatments. Subsequently, SMAD2, SMAD3, and SMAD4 expression levels in the DN cells model were significantly decreased by YLE treatments (5 to 50 µg/mL), while the SMAD7 expression level was increased. This result supported earlier research showing that YLE could lower TGF-β1 by inhibiting SMADs (37). Glomerulosclerosis and other diseases such as DN have pertained to reduced antioxidant defense, hence the enhancement of antioxidant ability prevents the pathogenesis (25). YLE has high antioxidant activities due to the large quantities of phenolics and flavonoids as a source of biofunctional compounds yacon leaves contain (20). It affirmed previous research that black soybean extract (*Glycine max* L. Merr) which contains high phenolic and flavonoid, exhibited antioxidant and anti-inflammatory activities (38,39) and antifibrosis by downregulating fibronectin, TGF-β1 levels on CKD cells model, antioxidant by decreasing ROS level on CKD cells model (24).

In summary, this study elucidated YLE therapeutic effects against the DN cells model. The treatments attenuated DN through the obstructions of mesangial proliferation, oxidative stress, and renal inflammation. The lowered MDA levels signify the YLE effect as an antioxidant. Moreover, the decreases of TGF-β1, TNF-α, SMAD2, SMAD3, SMAD4, and the increase of SMAD7 indicated anti-inflammatory and antifibrotic activities of YLE. Therefore, YLE (5 to 50 µg/mL) promises a role as anti-DN (Fig. 4).

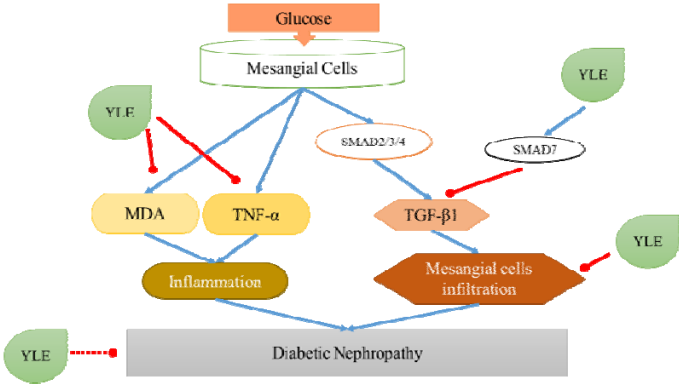

Fig. 4. Proposed antifibrotic, anti-inflammatory, and antioxidant mechanisms of YLE toward DN. Inflammation was finally brought on by glucose induction toward mesenchymal cells, which generated DN and was indicated by an increase in TNF-α, TGF-β1, and MDA. The levels of TNF-α, TGF-β1, and MDA were decreased by YLE. As a result, SMAD2, SMAD3, and SMAD4 were reduced and SMAD7 was elevated, preventing inflammation and alleviating DN. DN, Diabetic nephropathy; YLE, Yacon leaves extract; TGF, transforming growth factor; TNF, tumor necrosis factor; MDA, malondialdehyde; SMAD, small mothers against decapentaplegic. ——● indicates inhibition, ·····● potency of inhibition, and —→ stimulation.

CONCLUSION

YLE has potential to reduce mesangial cell proliferation and the levels of TGF-β1, TNF-α, and MDA in diabetic nephropathy cell models. The treatment of YLE was also able to downregulate the expressions of SMAD2, SMAD3, and SMAD4 genes, upregulate SMAD7 gene expression and reduce cell proliferation in diabetic nephropathy cell models. This research implies that YLE has potential as renal antifibrosis, anti-inflammatory, and antioxidant as well as anti-DN.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

W. Widowati contributed to the conceptualization, methodology, resources, data collection and validation, writing, reviewing, and editing of the article. R. Tjokropranoto and P. Onggowidjaja contributed to conceptualization, resources, and validation. H.S.W. Kusuma contributed to resources, validation, writing, and revising the article. C.R. Wijayanti, M. Marthania, and A. Yati contributed to the investigation, data collection, writing, and editing of the article. R. Rizal contributed to conceptualization, methodology, and validation. The finalized manuscript was approved by all authors.

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