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Research Paper

Potential of Bitter Melon (*Momordica charantia* L.) Extract for Chronic Kidney Disease Based on In Vitro Study via TGF/SMADs Signaling, Antioxidant, Antiinflammation, Apoptosis Inducer Activities

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Abstract
Chronic kidney disease (CKD) is a physiological abnormality in the kidneys whose prevalence is expected to continue to increase. On the other hand, Bitter melon (*Momordica charantia* L.) is known to have the potential to manage CKD. This study explores the compound content of *M. charantia* ethanol extract (MCEE) and its potential for CKD based on in vitro assays. To model chronic kidney disease (CKD), SV40 MES-13 (mouse glomerular mesangial) cells were exposed for 3 days to 20 mM glucose. After glucose induction, the cells were subjected with different concentrations of MCEE (*Momordica charantia* L. ethanolic extract). The chemical profile of MCEE was analyzed using LC/MS-MS. Cell viability was examined through the WST assay, while intracellular ROS and apoptosis levels were measured by flowcytometry. Colorimetry was used to analyze SOD, MDA, and CAT levels. ELISA was used to analyze inflammatory proteins (TGF-β1, IL-6, TNF-α, IL-1β) levels. Meanwhile, the relative gene expression of SMAD-2, SMAD-3, SMAD-4, SMAD-7 was examined through qRT-PCR. The results exhibited that MCEE contains cucurbitane p-coumaric, ferulic acid, caffeic acid, gallic acid, chlorogenic acid, and epicatechin. MCEE was also known to be non-toxic to SV40 MES-13 cells. In addition, MCEE reduced intracellular ROS levels, MDA, necrosis levels, and inflammatory proteins, while also regulating SMAD-2, SMAD-3, and SMAD-4 gene expression. MCEE increased levels of CAT, and SOD, and regulated SMAD-7 gene expression in the CKD cells model. The most effective MCEE is MCEE 50 μg/mL. MCEE demonstrated potential as a CKD treatment based on in vitro studies through TGF/SMADs signaling activity, antioxidant, anti-inflammatory, and apoptosis inducer.

Keywords
Anti-inflammatory, Antioxidant, Apoptosis, Bitter Melon, Chronic Kidney Disease

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1. INTRODUCTION

Chronic kidney disease (CKD) is a chronic ailment of impaired kidney function and decreased glomerular filtration rate, influenced by factors including diabetes mellitus (DM), chemotherapy, and kidney disease (Zeba et al., 2020; Ghelichi-Ghojogh et al., 2022). CKD affects >10% global population, primarily in individuals with DM and hypertension (Kovesdy, 2022). Diabetic glomerulosclerosis occurs due to hyperglycemia resulting in the accumulation of fibronectin in the renal glomerulus (Klemis et al., 2017). This process is one of the mechanisms for the occurrence of CKD in DM sufferers.

The incidence rate of CKD is 10.6% (stages 3-5) and 13.4% (stages 1–5), according to a meta-analysis of 6,908,440 individuals, with stage 5 associated with severe kidney damage (Hill et al., 2016; Kimura et al., 2018). Hustrini et al. (2022) study revealed CKD prevalence in Indonesia at 0.5% among 389,093 participants, focusing on young individuals aged 18-59. CKD, a disease-causing death, ranked 13th in 2016 and 12th in 2017 with the possibility will rank 5th globally in 2040 according to Global Regional and National (GBD) data Foreman et al. (2018); Bikbov et al. (2020), In Africa, Australia, Europe, Asia, Latin America, North America, Israel, Singapore, and Greece,

CKD is the primary cause of death.

Elevated mesangial cell glucose levels increase Reactive Oxygen Species (ROS), that may lead to the progression of CKD and cell membrane damage, characterized by increased MDA (Rasool et al., 2017). Superoxide dismutases (SOD) and catalase (CAT) are enzymatic antioxidants that play a crucial role in Reactive Oxygen Metabolites (ROM), converting reactive molecules into less reactive ones (Zaigham et al., 2015). Oxidative stress triggers an inflammatory response with a rise in inflammatory mediators including Interleukin (IL)-6, IL-1β, Tumor Necrosis Factor-α (TNF-α), and Transforming Growth Factor β-1 (TGF-β1) due to necrosis (Chen et al., 2018), which is the process of releasing molecules which triggers immunogenic and inflammatory responses that are distinct from apoptosis. Inhibiting necrosis and inflammation may be a treatment strategy. One important mediator in renal fibrosis is TGF-β1, which contributes to glomerular injury and proliferation of the mesangial matrix (Chen et al., 2018; Prahastuti et al., 2019b). Furthermore, Chen et al. (2018) This research highlight the important role of SMAD in both positive and negative signaling pathways triggered by the TGF-β superfamily. Short-term antidepressant drugs (SMADs) are categorized into (SMAD-4), R-SMADs (SMAD-1, -2, -3, -5, -8), inhibitory SMADs (SMAD -6 and -7). Studies show TGF-β modulates the SMADs signaling pathway, affecting the etiology and progression of CKD through processes like acetylation, sumoylation, ubiquitination, phosphorylation, and protein interactions (Zhang et al., 2015).

CKD treatment involves a complex process to prevent complications and side effects like accelerated kidney function loss (Whittaker et al., 2018). Diuretic drugs, commonly used in advanced stages of CKD, can cause hyperuricemia, glucose intolerance, and hyperlipidemia when used at higher doses. In addition, multidrug administration can cause adverse effects on patients. A study analyzing 200 elderly CKD patients in stages 3, 4, and 5 CKD found that 29.5% experienced QTC interval prolongation due to drug combinations, with Amiodarone, citalopram, and ciprofloxacin being the most dangerous (Sommer et al., 2020). Therefore, alternative treatments are necessary.

Momordica charantia L. is a widely cultivated member of the *Cucurbitaceae* family, especially in the Asian region (Wang et al., 2017; Huang et al., 2020). With various potential effects including antioxidant and antidiabetic (Fachinan et al., 2017), anti-inflammatory (Liaw et al., 2015; Liao et al., 2022), antiretroviral (Gupta et al., 2015), and antitumor (Fang et al., 2019). Fachinan et al. (2017) showed that fruit juice has type 1 antidiabetic activity through immunosuppressive activity and inducing T-helper cells. Apart from that, Gupta et al. (2015) showed that the seeds, fruit, leaves, and stems of *M. charantia* L. by in vitro study could reduce blood glucose levels in DM patients.

This study investigated the potential and chemical composition of *M. charantia* ethanol extract (MCEE) for CKD based on LC/MS-MS, cell viability, levels of (ROS, SOD, MDA, and CAT), inflammatory proteins levels (TGF-β1, IL-6, TNF-α, IL-1β), relative gene expression (SMAD-2, -3, -4, -7) in SV40 MES-13 cells (glucose-induced) as a CKD cells model.

2. EXPERIMENTAL SECTION

2.1 Materials

Materials used were SV40 MES-13 (ATCC®CRL-1927), Minimum Essential Medium (MEM) medium (Biowest, L0416-500, France), Water-soluble tetrazolium (WST-8) test (Elabscience, E-CK-A362, United States), ROS Fluorometric Assay Kit (Elabscience, E-BC-K138-F, United Stated), Trypsin-EDTA (Biowest, L0931-100, France), SOD Kit (Elabscience, E-BC-K020-M, United States), MDA Kit (Elabscience, E-BC-K025-M, United States), CAT Kit (Elabscience, E-BC-K031-M, United States), Apoptosis Detection Kit (Elabscience, ECK-A211, United States), Mouse ELISA Kit TGF-β1 levels (Elabscience, E-EL-0162, United States), IL-6 (Elabscience, E-EL-M0036, United States), TNF-α (Elabscience, E-EL -M3063, United States), and IL-1β (Elabscience, E-EL-M0044, United States).

2.2 Methods

2.2.1 Extraction Preparation of *M. charantia L.*

Fruit *M. charantia* L. extract was processed at PT Industri Jamu Borobudur (Batch No. 009PU1.3) according to Good Traditional Medicine Manufacturing Methods (CPOTB). Extraction was carried out using 70% ethanol with maltodextrin as excipient (Widowati et al., 2023b).

2.2.2 LC/MS-MS

The compound content contained in MCEE was analyzed using LC/MS-MS (Acella 1250, TSQ Quantum Access Max). A column with Hypersil Glod was used for analysis. With electrospray ionization (ESI), the TSQ Quantum Access MS/MS mass spectrometer with triple quadrupole configuration is operated with a positive charge (Widowati et al., 2017; Cserbik et al., 2023; Priyandoko et al., 2023).

2.2.3 SV40 MES-13 Cell Culture and CKD Treatment

SV40 MES-13 cell lines were procured from the Aretha Medika Utama, Indonesia. SV40 MES-13 cell culture procedure was based on Widowati et al. (2022, 2023a) with modification. SV40 MES-13 cell culture was carried out using a complete Minimum Essential Medium (MEM) medium. The CKD model was carried out by inducing cells using glucose at a concentration of 20 mM for 3 days. CKD treatment was carried out with several treatments including Negative Control (NC), which contained of untreated cells; Positive Control (PC), which contained of SV40 MES-13 cells induced by glucose without MCEE treatment; DMSO control, which consisted of PC+DMSO 1% treatment; MCEE treatment groups, which consisted of PC+MCEE at different concentrations (3.13; 12.5; 50) μg/mL, MET was PC+Metformin 12.5 μg/mL.

Table 1. RNA Concentration & Purity

Sample	Concentration (ng/ μl)	Purity (λ260/ λ280 nm)
I (Negative Control)	149.50	2.0858
II (Positive Control)	229.40	2.1855
III (PC + DMSO 1%)	151.80	2.0660
IV (PC + MCEE 3.13 μg/mL)	171.30	2.0289
V (PC + MCEE 12.5 μg/mL)	188.70	2.1005
VI (PC + MCEE 50 μg/mL)	198.80	2.0463
VII (PC + Metformin 12.5 μg/mL)	137.80	1.9170

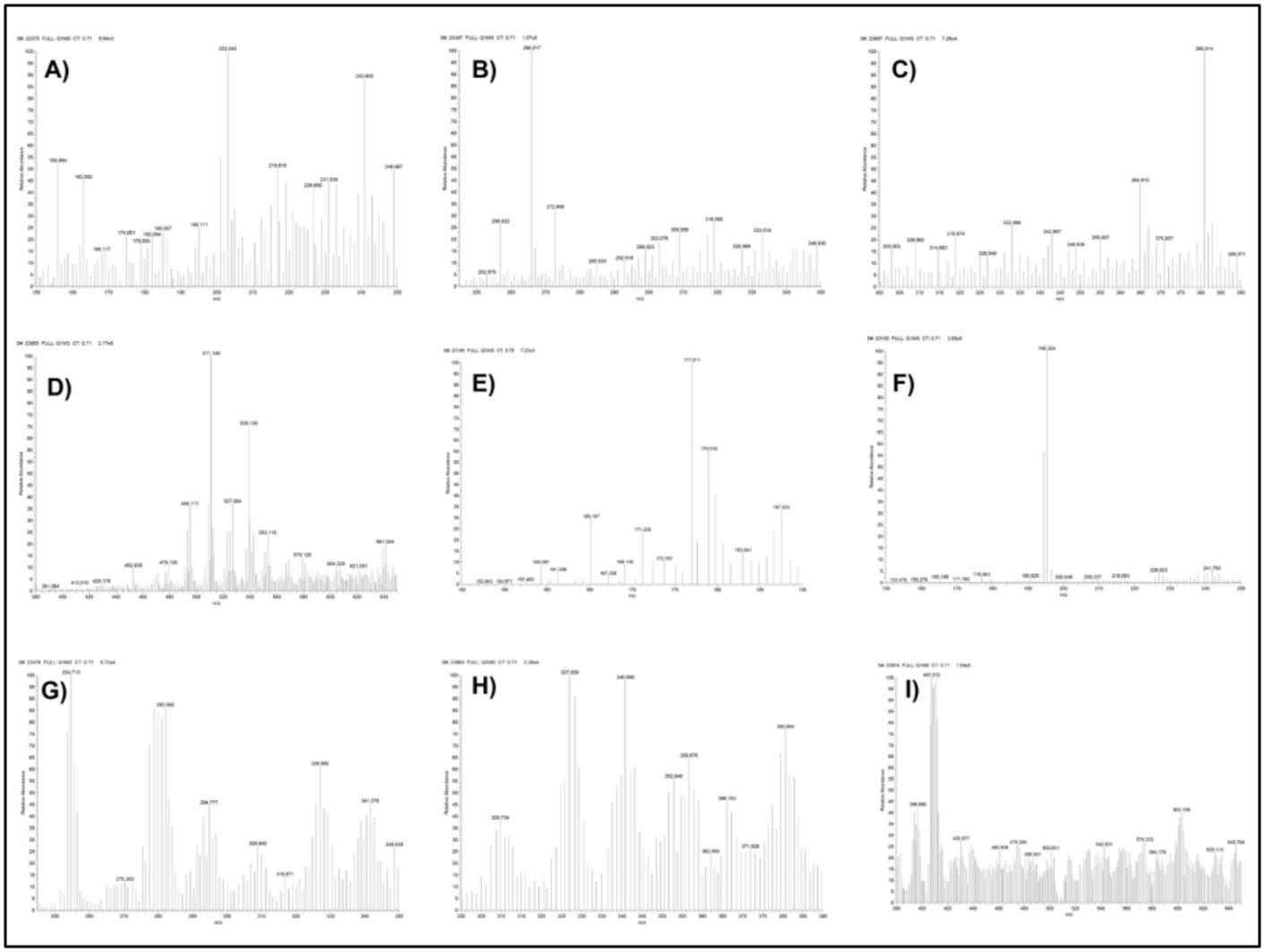

Figure 1. LC/MS-MS spectrum of MCEE

***** (A) Mass spectra 150-250 with positive ionization, (B) mass spectra 250-350 with positive ionization, (C) mass spectra 300-390 with positive ionization, (D) mass spectra 380-650 with positive ionization, (E) mass spectra 150-190 with negative ionization, (F) mass spectra 150-250 with negative ionization, (G) mass spectra 250-350 with negative ionization, (H) mass spectra 300-390 with negative ionization, (I) mass spectra 380-650 with negative ionization

2.2.4 Cell Viability Assay

Cells are cultured in 96-well plates. previously using MEM complete medium 10% FBS then the next day, the cell culture medium was replaced, and glucose induction was carried out for 3 days (37°C, 5% CO_2). Following three days, 180 μL of 2% FBS complete culture media was used to replace the culture

Table 2. Primary Design

Gene	Forward (5'-3') Reverse (5'-3')	Product size (bp)	Annealing (°C)	Cycle	References
SMAD 2 (mouse)	F: ATTACATCCCAGAAACACCAC R: TAGTATGCGATTGAACACCAG	196	59	40	NM_001252481.1
SMAD 3 (mouse)	F: GTAGAGACGCCAGTTCTACCT R: CATCTTCACTCAGGTAGCCAG	178	59	40	NM_016769.4
SMAD 4 (mouse)	F: GAGAACATTGGATGGACGAC R: ACATACTTGGAGCATTACTCTG	242	54	40	NM_001364967.1
SMAD 4 (mouse)	F: ACTCTGTGAACTAGAGTCTCCC R: CTCTTGGACACAGTAGAGCCT	241	59	40	NM_001042660.1
β-actin (mouse)	F: TCAAGATGGTGAAGCAG R: ATGTAGGCCATGAGGTCCAC	217	59	40	NM_001289726

Table 3. Identification of Target Compound in MCEE Using LC/MS-MS

Compound content in MCEE	MW (g/mol)	MS $[M+H]^+$	$[M+NH4]^+$	$[M+Na]^+$	$[M+K]^+$	$[M-H]^-$	$[M+Na-2H]^-$	$[M+Cl]^-$	$[M+K-2H]^-$
Gallic acid	170.022	171.029	188.055	193.011	208.985	169.014	190.996	204.991	206.970
p-coumaric acid	164.047	165.054	182.081	187.037	203.011	163.040	185.022	199.017	200.996
Ferulic acid	194.057	195.065	212.092	217.047	233.021	193.051	215.033	229.027	231.007
Caffeic acid	180.042	181.049	198.076	203.031	219.005	179.035	201.017	215.012	216.991
Cucurbitane	414.422	415.430	432.456	437.412	453.386	413.415	435.397	449.392	451.371
Chlorogenic acid	354.095	355.102	372.129	377.084	393.058	353.088	375.070	389.064	391.044
Epicatechin	290.079	291.086	308.113	313.068	329.042	289.072	311.054	325.048	327.028

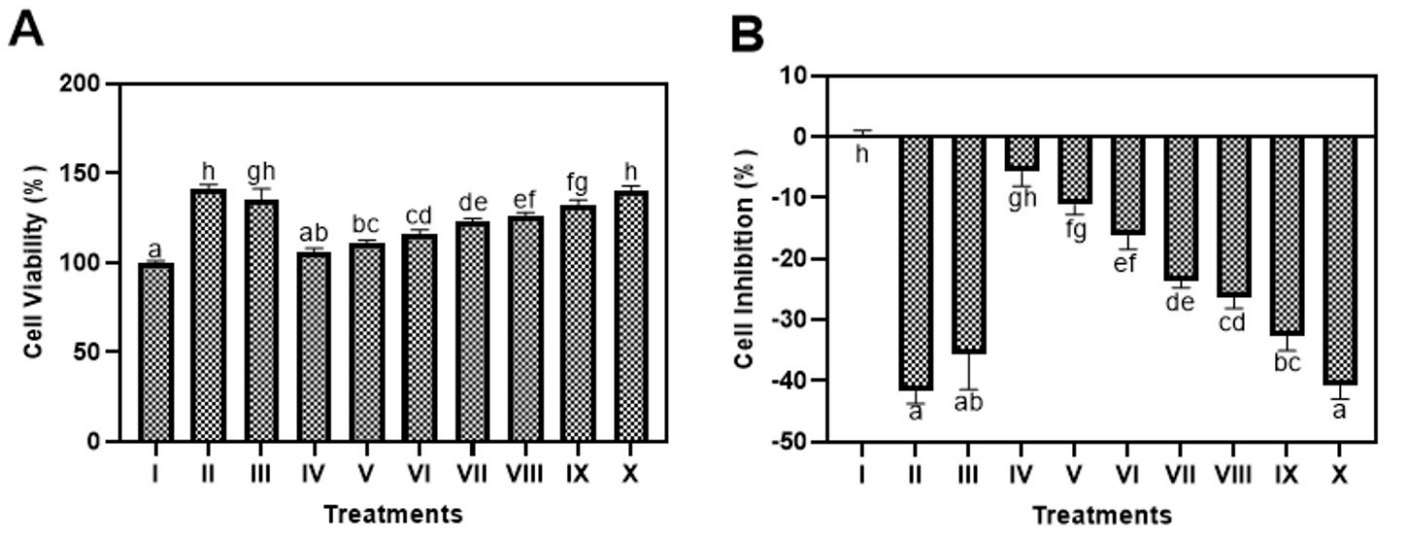

Figure 2. Effect Various Concentrations of MCEE Toward Cells Viability and Inhibition in SV40 MES-13 Cells

***** Cell Viability (A) and Cell Inhibition (B). Different superscript marks indicate significant differences based on Dunnett's T3 post hoc test ($p<0.05$). I : NC (negative control, untreated SV40 MES-13 cells), II: PC (positive control: SV40 MES-13 cell induced by glucose without MCEE treatment), III: DMSO (PC+DMSO1%), IV: (PC+MCEE 3.13μg/mL), V: (PC+MCEE 6.25 μg/mL), VI: (PC+MCEE 12.5 μg/mL), VII: (PC+MCEE 25 μg/mL), VIII: (PC+MCEE 50 μg/mL), IX: (PC+MCEE 100 μg/mL), X: (PC+MCEE 200 μg/mL)

medium. 20 μL of MCEE was then added at several doses (200, 100, 50, 25; 12.5; 6.25; 3.125) μg/mL, and the mixture was cultured for 24 h. Using an improved reagent containing resazurin in a water-soluble tetrazolium (WST-8) test. Next, the 3 concentrations are selected and used for further testing.

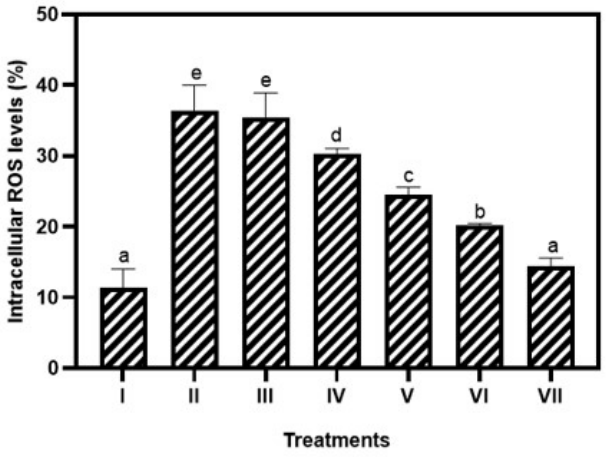

Figure 3. Effect Various Concentrations of MCEE Toward Intracellular ROS Level in SV40 MES-13 Cells

*Different superscript marks present significant differences based on Dunnet T3 post hoc ($p<0.05$). I: NC (negative control, untreated SV40 MES-13 cells), II: PC (positive control, SV40 MES-13 cell induced by glucose without MCEE treatment), III: DMSO (PC+DMSO1%), IV:(PC+MCEE 3.13 μg/mL), V:(PC+MCEE 12.5 μg/mL), VI:(PC+MCEE 50 μg/mL), VII: (PC+Metformin 12.5 μg/mL)

2.2.5 Intracellular ROS Level Assay

SV40 MES-13 cells' intracellular ROS levels were measured by ROS Fluorometric Assay Kit, following the guidelines (Widowati et al., 2014; Prahastuti et al., 2019b; Baris et al., 2023). In the experimental group, the Negative Control (NC) consisted of untreated cells, whereas the Positive Control (PC) comprised SV40 MES-13 cells induced by glucose. DMSO control was PC+DMSO 1% treatment, MCEE was PC+MCEE with different concentrations (3.13; 12.5; 50) μg/mL, MET was PC+Metformin 12.5 μg/mL added 1mL DCFH-DA 10 μM (incubation 60 minutes), the cells subsequently detached using a solution of 0.25% Trypsin-EDTA and the cell pellet was then rinsed using serum-free medium. MACSQuant 10 flow cytometry (Miltenyi) was employed to measure ROS levels, utilizing the FITC fluorescent dye.

2.2.6 SOD, MDA, and CAT Level Assay

SOD, MDA, and CAT levels were analyzed based on Widowati et al. (2023b) research with modifications. SOD, MDA, and CAT Kit is used in research following the instructions from the manufacturer.

2.2.7 Apoptosis Percentage (Live Cell, Necrosis, Early, and Late Apoptosis) Assay

With modifications, the percentage of SV40 MES-13 cell death was analyzed based on Priyandoko et al. (2024) research. Each treatment was measured using the Apoptosis Detection Kit according to the guidelines. Cell apoptosis was analyzed using MACSQuant 10 flow cytometry.

2.2.8 TGF-β1, IL-6, TNF-α, and IL-1β Levels Assay

Supernatant cells that have been treated are harvested and tested using an ELISA kit to determine the inflammatory protein levels (TGF-β1, IL-6, TNF-α, IL-1β) in SV40 MES 13 cells. Mouse ELISA Kit were used to measured TGF-β1, IL-6, TNF-α, IL-1β levels, following the guidelines provided by the manufacturer (Widowati et al., 2022). The absorbance was read employing microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, USA).

2.2.9 SMAD2, SMAD3, SMAD4, and SMAD7 Expression Gene Assay

The stages of gene expression analysis include RNA isolation, cDNA synthesis, and qRT-PCR according to the protocol of Widowati et al. (2022). Table 1 displays the concentration and RNA purity and the primer sequence (Macrogen) shown in Table 2. β-actin is used as a housekeeping gene.

2.2.10 Statistical Analysis

Statistical analysis was conducted following Hidayat et al. (2022) using the software of Statistical Package for Social Sciences (SPSS) version 16. Data represented as average ± standard deviation from three repetitions.

3. RESULTS AND DISCUSSION

3.1 Compound analysis in MCEE by LC/MS-MS

Analysis of the results compound content in MCEE using LC/MS-MS is depicted in Figure 1 and Table 3. The results show that MCEE was identified as containing several compounds including p-coumaric, gallic acid, ferulic acid, caffeic acid, cucurbitane, chlorogenic acid, and epicatechin.

Herbal ingredients have the potential to be used in the treatment of CKD because of their affordable cost and low side effects (Gautam et al., 2021). *M. charantia* L. can play a role as antioxidant, antidiabetic, antitumor, antiretroviral, and anti inflammatory. The LC/MS-MS analysis results show that MCEE was identified as containing several compounds such as ferulic acid, gallic acid, p-coumaric, chlorogenic acid, caffeic acid, cucurbitane, epicatechin (Figure 1 and Table 3). These outcomes are consistent with earlier studies which stated that bitter melon extract contains the compounds gallic acid, p-coumaric, ferulic acid, caffeic acid, cucurbitane, chlorogenic acid, and epicatechin (Raina et al., 2016; Sathasivam et al., 2021). Phenolic acids have been shown to have anti-fibrotic, antioxidant, and anti-inflammatory properties that contribute to the control of blood sugar levels and cholesterol levels (Jin et al., 2023). For example, compared to similar substances, gallic acids are known to have potent antioxidant properties toward OH, OOH radicals, and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (Marino et al., 2014; Malinda et al., 2017). Other studies show anti-inflammatory properties of gallic acid (Bai et al., 2021; Li et al., 2023).

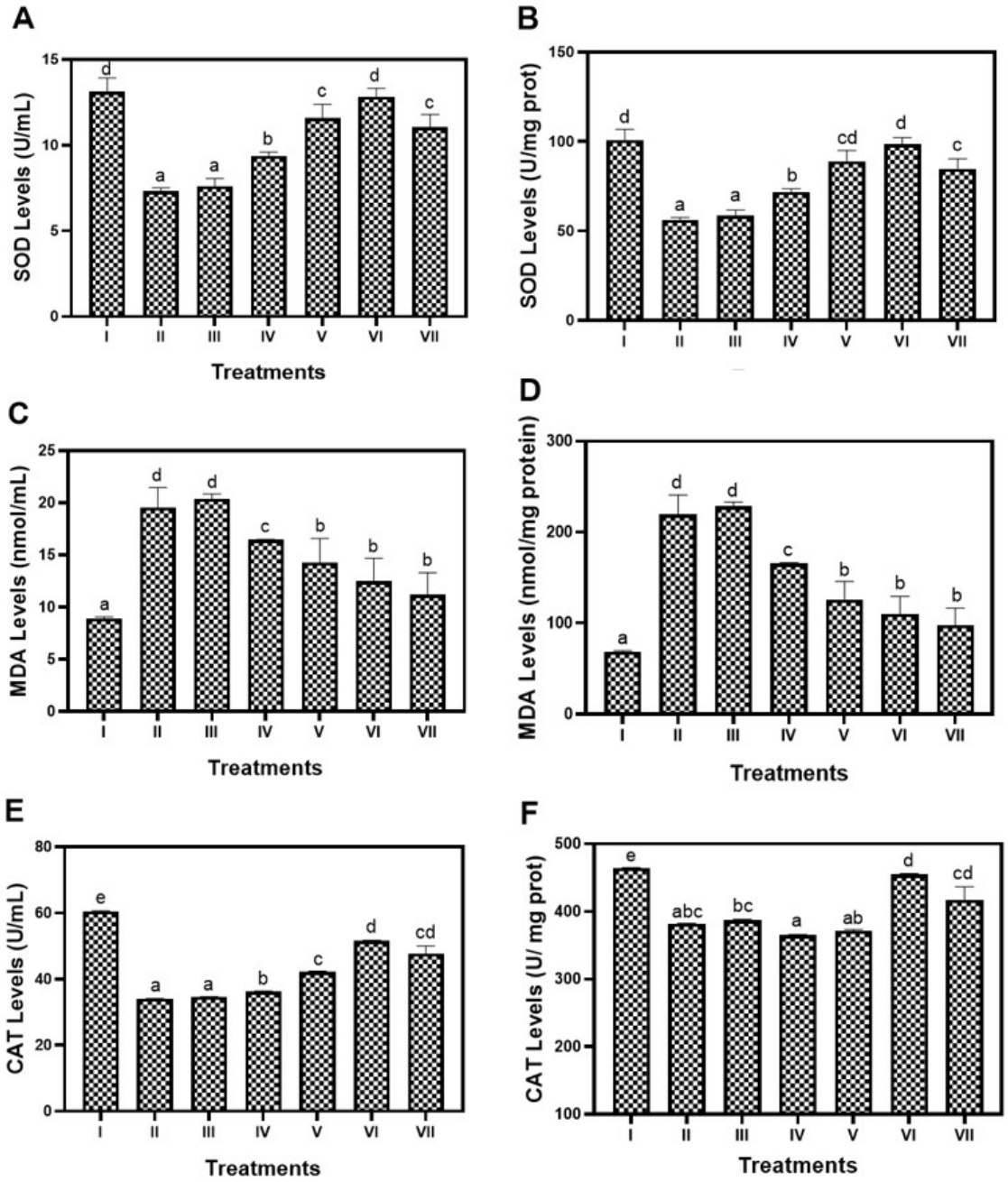

Figure 4. Effect from Various Concentrations of MCEE Toward SOD, MDA, and CAT Levels in SV40 MES-13 Cells

*SOD Levels (U/mL) (A), SOD Levels (U/mg prot) (B), MDA Levels (nmol/mL) (C), MDA Levels (nmol/mgprot) (D), CAT Levels (U/mL) (E), CAT Levels (U/mg prot) (F). Different superscript marks present significant differences based on One Way ANOVA Tukey HSC and Dunnet T3 post hoc ($p<0.05$). I: NC (negative control, untreated SV40 MES-13 cells), II : PC (positive control, SV40 MES-13 cell induced by glucose without MCEE treatment), III: DMSO (PC+DMSO1%), IV: (PC+MCEE 3.13μg/mL), V: (PC+MCEE 12.5μg/mL), VI: (PC+MCEE 50μg/mL), VII:(PC+Metformin 12.5μg/mL)

3.2 Effect of MCEE toward Cell Viability in SV40 MES-13 Cells

Figure 2A-B showed MCEE on the viability and inhibition of SV40 MES-13. In the PC group showed significant improvement in SV40 MES-13 cell viability (141.73%) compared to NC (100%) due to cell swelling due to glucose induction. MCEE treatment is known to reduce cell viability and increase cell inhibition with concentrations that show significantly different results from PC, namely MCEE (3.13; 6.25; 12.5; 25; 50) μg/mL. The results show that MCEE is not toxic to SV40

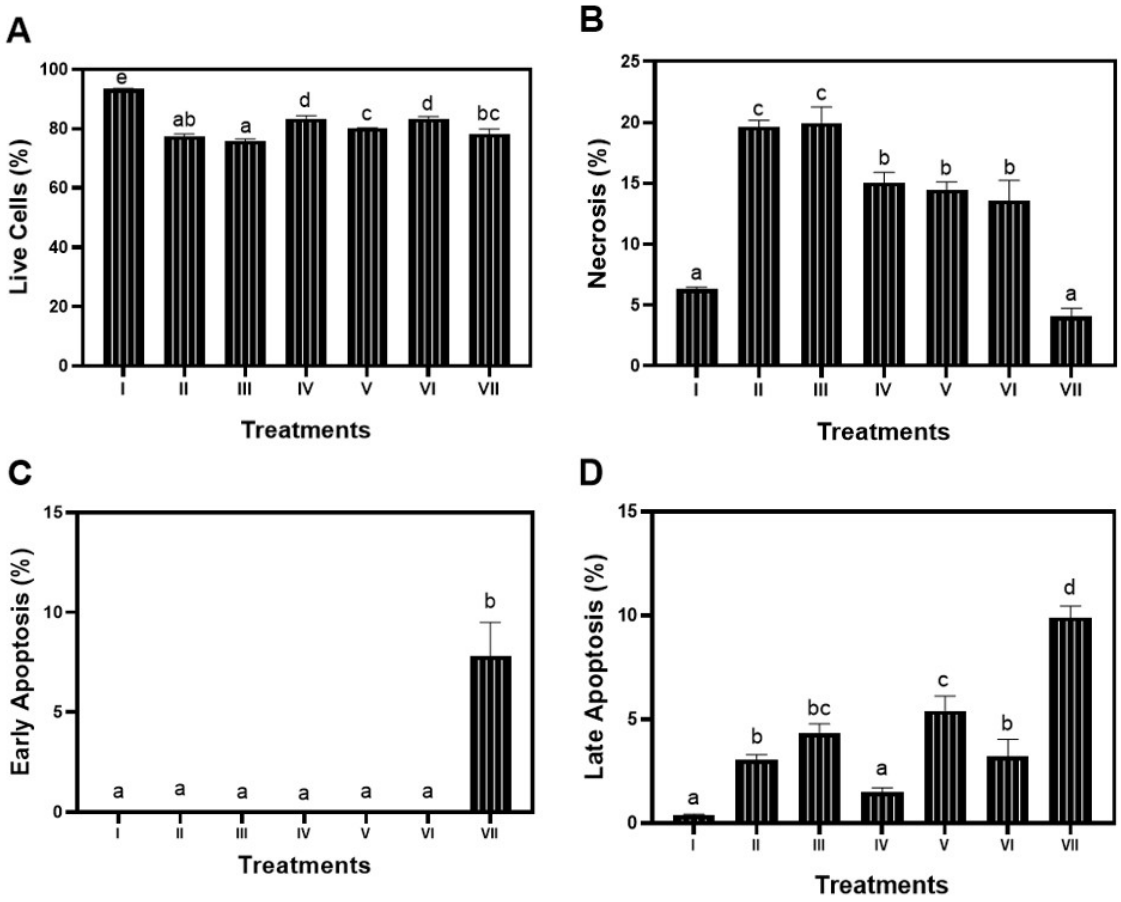

Figure 5. Effect Various Concentrations of MCEE Toward Apoptosis Percentage in SV40 MES-13 Cells

*Live Cells (%) (A),Necrosis (%) (B), Early Apoptosis (%) (C), Late Apoptosis (%) (D). Different superscript marks present significant differences according to One Way ANOVA Tukey HSD ($p<0.05$). I: NC (negative control, untreated SV40 MES-13 cells), II: PC (positive control, SV40 MES-13 cell induced by glucose without MCEE treatment), III: DMSO (PC+DMSO1%), IV: (PC+MCEE 3.13 μg/mL), V : (PC+MCEE 12.5 μg/mL), VI: (PC+MCEE 50 μg/mL), VII: (PC+Metformin 12.5 μg/mL)

MES-13 cells which is indicated by no decrease in cell viability which is lower than NC.

CKD is increasing in both incidence and prevalence in society (Yang et al., 2020). This disease occurs when kidney function is disrupted, thereby reducing kidney performance. This condition causes a buildup of metabolic waste in the kidneys that cannot be excreted by the body (Orr and Bridges, 2017). This condition occurs due to renal vasculopathy, fibrosis, augmented glomerulosclerosis, atrophy and scar tissue, and tubular inflammation (Rasool et al., 2017). The chronic kidney disease (CKD) main causative factors are hypertension and diabetes, while other factors commonly present in CKD patients in developing countries include exposure to toxins or heavy metals and HIV (Ekrikpo et al., 2018; Bikbov et al., 2020). In this study, the in vitro CKD model was developed by inducing SV40 MES-13 cells using glucose.

Based on the research results, MCEE treatment is known to reduce the viability of SV40 MES-13 cells which experience proliferation due to cell swelling due to glucose induction. However, MCEE is not toxic to SV40 MES-13 cells which is indicated by no decrease in cell viability which is lower than NC (Figure 2). This is because various MCEE components may influence several mechanisms in SV40 MES-13 cells in the CKD model, preventing cell swelling, which is characterized by increased cell viability in the PC group. Cell viability is an indicator of the number of healthy cells in the sample, which reflects the regulation of genes, proteins, and certain mechanisms that cause cells death or survival after exposure to toxic substances. agents and other abnormal conditions (Adan et al., 2016).

3.3 Effect of MCEE toward Intracellular ROS in SV40 MES-13 Cells

The findings presented that the intracellular ROS level was higher in the PC group (36.32%) in contrast to the NC group (11.37%) (Figure 3). MCEE treatment can reduce intracellular ROS levels compared to PC with the most effective MCEE concentration being MCEE 50μg/mL (20.15%).

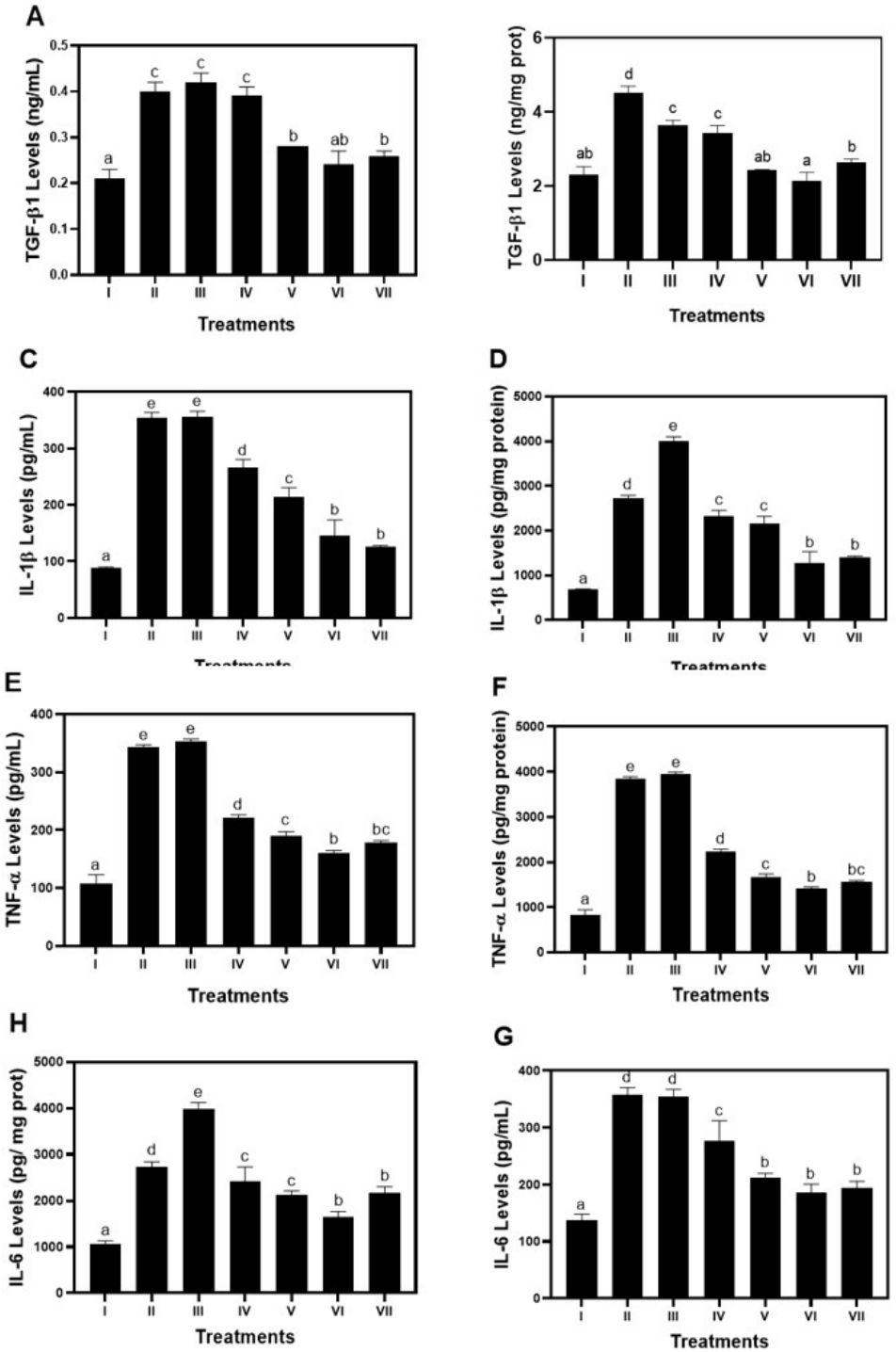

Figure 6. Effect Various Concentrations of MCEE toward TGF-β1, IL-1β, TNF-α, IL-6 Levels in SV40 MES-13 Cells

*TGF-β1 Levels (ng/mL) (A), TGF-β1 Levels (ng/mg prot) (B), IL-1β Levels (ng/mL) (C), IL-1β Levels (ng/mgprot) (D), TNF-α Levels (ng/mL) (E), TNF-α Levels (ng/mg prot) (F), IL-6 Levels (ng/mL) (G), IL-6 Levels (ng/mg prot) (H). Different superscript marks present significant differences based on One Way ANOVA Tukey HSD and Dunnet T3 post hoc test ($p<0.05$). I: NC (negative control, untreated SV40 MES-13 cells), II: PC (positive control, SV40 MES-13 cell induced by glucose without MCEE treatment), III: DMSO (PC+DMSO1%), IV: (PC+MCEE 3.13 μg/mL), V: (PC+MCEE 12.5 μg/mL), VI: (PC+MCEE 50 μg/mL), VII: (PC+Metformin 12.5μg/mL)

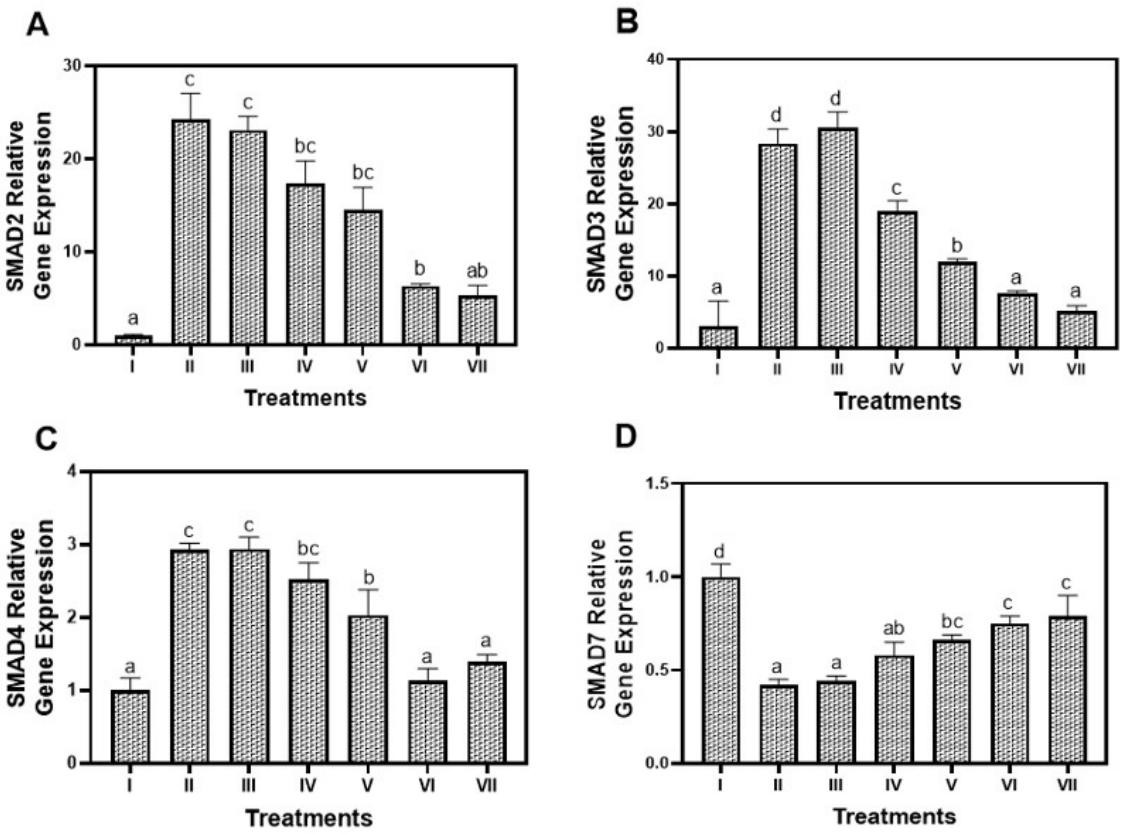

Figure 7. Effect Various Concentrations of MCEE Toward SMAD-2, -3, -4, -7 Relative Gene Expression in SV40 MES-13 Cells

*SMAD2 Relative Gene Expression (A), SMAD3 Relative Gene Expression (B), SMAD4 Relative Gene Expression (C), SMAD7 Relative Gene Expression (D). Different superscript marks present significant differences based on One Way ANOVA Tukey HSD ($p<0.05$). I: NC (negative control, untreated SV40 MES-13 cells), II: PC (positive control, SV40 MES-13 cell induced by glucose without MCEE treatment), III: DMSO (PC+DMSO1%), IV: (PC+MCEE 3.13μg/mL), V: (PC+MCEE 12.5μg/mL), VI: (PC+MCEE 50μg/mL), VII: (PC+Metformin 12.μ/mL

An uncontrolled increase in ROS can cause fibrosis and inflammation which ultimately causes CKD, in the process of which ROS formation can affect kidney function, including tubular sodium transport, medullary blood flow, and tubuloglomerular processes (Irazabal and Torres, 2020). MCEE treatment is known to reduce intracellular ROS levels with the most effective MCEE being 50 μg/mL (Figure 3). Several studies have found that reducing ROS levels can reduce the potential for CKD (Rapa et al., 2019). Prahastuti et al. (2019b) stated that antioxidants can convert ROM into harmless ones which then play a role in regenerating damaged extracellular matrix and protecting mesangial cells from glomerulosclerosis.

3.4 Effect of MCEE toward SOD, MDA, and CAT Levels in SV40 MES-13 Cells

In the PC group, there was a decrease in SOD (7.32U/mL; 56.11U/mg protein) and CAT (34.04U/mL; 381.87U/mg protein) levels and increased levels of MDA (19.55 U/mL; 219.30 U/mg protein) compared to NC (SOD 13.13 U/mL; 100.69 U/mg protein) (MDA 8.94U/mL; 68.55U/mg protein) (CAT 60.52U/mL; 464.02U/mg protein) (Figure 4). MCEE is known to be able to increase SOD and CAT levels and decrease MDA levels, with the most significant effect observed at MCEE 50 μg/mL (SOD 12.83U/mL; 98.37U/mg protein) (MDA 12.46U/mL; 109.75U/mg protein) (CAT 51.53 U/mL; 454.31U/mg protein). The results also showed that MCEE 50 μg/mL was the most effective in enlarging SOD and CAT levels and reducing MDA levels.

There is an imbalance between ROS production in the mitochondria and the antioxidant system in the body in CKD patients, in line with increased cell membrane damage as indicated by increased MDA levels Rasool et al. (2017), and a decrease in enzymatic endogenous antioxidants including SOD and CAT (Zaigham et al., 2015). MCEE treatment can increase CAT and SOD levels and reduce MDA levels, even statistical results show that MCEE can increase CAT levels and reduce MDA levels with results that are not significantly different from Metformin (Figure 4). MCEE 50 μg/mL is the most effective. Metformin, the pharmacological drug most commonly used in Type 2 DM, is effective in lowering glucose levels, but

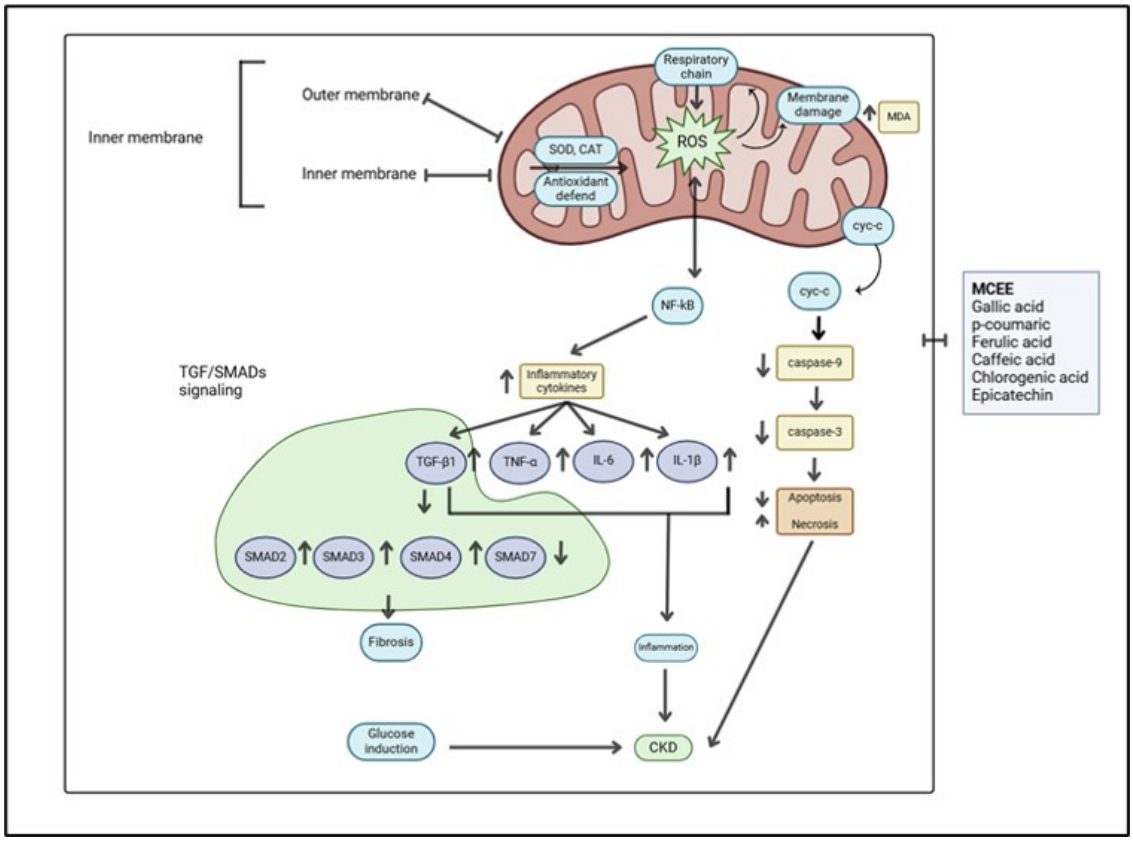

Figure 8. Impact of MCEE on Endogenous Pathways in a CKD Model Using Glucose-Induced SV40 MES-13 Cells

*Increased ROS formation in mitochondria of CKD causes an imbalance of intracellular antioxidants SOD and CAT and causes membrane damage characterized by increasing MDA. This process causes a decrease in apoptosis which is characterized by a decrease in casp-9 and casp-3. On the other hand, NF-KB activation causes an increase in inflammatory cytokines such as TGF-β1, IL-1β, TNF-α, and IL-6 which then activates the TGF/SMADs signaling pathway which then causes fibrosis. MCEE has potential as a CKD treatment in inhibiting this mechanism

it can potentially cause lactic acidosis, heart failure, and liver disorders (Flory and Lipska, 2019). The effect of MCEE on SOD, CAT, and MDA levels caused by the presence of phenolic acid and chlorogenic acid. This statement is supported by Qi et al. (2020) where in an in vivo study of diabetic nephropathy model mice, phenolic acids could prevent oxidative stress, inflammation, and fibrosis via lowering MDA levels and raising CAT and SOD levels. In addition, chlorogenic acid can reduce MDA levels and increase SOD levels (Bao et al., 2018; Zhou et al., 2019).

3.5 Effect of MCEE toward Apoptosis Levels in SV40 MES-13 Cells

The findings demonstrated that, in comparison to NC (6.31%), glucose induction in SV40 MES-13 cells (PC) (19.62%) experienced a significant increase cell necrosis (Figure 5). MCEE treatment could reduce the level of necrosis in cells and induce apoptosis without affecting SV40 MES-13 live cells. The most optimal treatment to reduce necrosis is MCEE with a concentration of 50 μg/mL (13.59%).

Accumulation of ROS can cause mitochondrial damage (Sun et al., 2020), which ultimately affects ATP synthesis and cytochrome-c release which then causes glomerular and tubular damage, and increased necrosis (Gong et al., 2019; Zhu et al., 2022). MCEE can reduce the level of necrosis and induce apoptosis without affecting SV40 MES-13 live cells; MCEE 50 μg/mL is the most effective concentration (Figure 5). Inhibition of necrosis is important in the treatment of CKD (Landau et al., 2019). Necrosis is non-programmed cell death and is often associated with extensive tissue damage and causes increased inflammation.

3.6 Effect of MCEE toward TGF-β1, IL-1β , TNF-α, and IL-6 in SV40 MES-13 Cells

Figure 6 shows the effects of MCEE on inflammatory proteins (TNF-α, IL-1β, TGF-β1, and IL-6) in SV40 MES-13 cells. Results show an increase in inflammatory protein levels after glucose induction (PC) versus NC. Administration of MCEE reduced inflammatory protein levels with the most effective was MCEE 50 μg/mL. The statistical findings indicate that

subtraction does not show a significant difference inflammatory protein levels between Metformin and MCEE 50 μg/mL.

These pro-inflammatory cytokines and chemokines include increases in TGF-β1, IL-1β, IL-6, TNF-α. The results showed an increase in inflammatory protein levels in the positive control group (glucose induction). However, MCEE treatment can reduce inflammatory protein levels with MCEE 50μg/mL being the most effective concentration with statistical results showing that the effect of MCEE 50μg/mL is not significantly different from Metformin (Figure 6). This Caused by the presence of phenolic acid. content in MCEE. Chowdhury et al. (2019) in vivo on DM mouse models showed that 50 mg/kg bw phenolic acid could treat CKD by inhibiting inflammatory protein levels via the Nuclear Factor Kappa B (NF-KB)-mediated inflammatory pathway. Research by Bao et al. (2018) also showed that chlorogenic acid can inhibit inflammation by reducing TNF-α, IL-6, and IL-1β through the NF-E2-related factor 2 (Nrf2/HO-1) and NF-KB pathways. In addition, antioxidants are known to downregulate TGF-β1 expression and fibronectin genes Han et al. (2017), by reducing the expression of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidases p47phox and p22phox which are sources of oxidative stress (Widowati et al., 2018).

3.7 Effect MCEE toward SMAD-2, -3, -4, -7 Relative Gene Expression in SV40 MES-13 Cells

The effect of MCEE on SMAD-2, -3, -4, -7 relative gene expression can be seen on Figure 7. In the PC group, there was an upregulation of SMAD-2 (24.20), SMAD3 (28.36), and SMAD4 (2.93) gene expression and a downregulation of SMAD7 gene expression (0.42) versus NC (1.01) (1.01) (1.01) (1.00). The results showed that 50 μg/mL MCEE treatment significantly compared to other MCEE concentrations, was known to downregulate SMAD2 (6.34), SMAD3 (7.52), and SMAD4 (1.13) gene expression and upregulate SMAD7 gene expression (0.75).

SMADs have a role in the pathological process of fibrogenesis, which contributes to the mechanism and progression of CKD. SMAD signaling itself is influenced by the TGF-β superfamily. From several groups, SMAD-2, -3, and -4 act as profibrotic agents, while SMAD7 acts as a protective agent with antifibrotic properties (Ma and Meng, 2019). The results showed that MCEE 50 μg/mL could significantly prevent kidney fibrosis which causes CKD by downregulating SMAD-2, -3, and -4 gene expression and upregulating SMAD7 gene expression (Figure 7). The flavonoid and phenolic acid content in MCEE causes this effect. The content of flavonoids and phenolic acids can act as an anti-inflammatory, antioxidant (Widowati et al., 2019; Prahastuti et al., 2020), and anti-fibrosis by reducing TGF-β1 levels and regulating fibronectin in CKD model cells (Prahastuti et al., 2019a). The mechanism of MCEE as anti-chronic kidney disease shown in Figure 8.

4. CONCLUSIONS

MCEE is known to contain gallic acid, ferulic acid, p-coumaric, caffeic acid, chlorogenic acid, cucurbitane, and epicatechin based on LC/MS-MS analysis. Based on in vitro studies, MCEE is known to be non-toxic to SV40 MES-13 cells and has potential as a CKD treatment through TGF/SMADs signaling activity, antioxidant, anti-inflammatory, and apoptosis inducer with effects including reduction of intracellular ROS levels, MDA levels, necrosis levels, and protein expression (TGF-β1, IL-1β, TNF-α, IL-6), regulation of gene expression (SMAD2, SMAD3, SMAD4) and can increase levels of SOD, CAT, and SMAD7 gene expression regulation in SV40 MES-13 cells as a CKD model.

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