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A Promising Functional Food for Diabetes Prevention, Antioxidation, and Anti-inflammation of Green Coffee Bean Extract

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Abstract

Functional foods and nutrition have become increasingly popular in preventing and reducing the incidence of diabetes. Green coffee bean extract (GCBE) has received much interest because of the evidence that coffee consumption reduces the risk of diabetes and many inflammatory diseases. This study was designed to investigate the phytochemicals contained in GCBE and their antioxidant, anti-diabetic, and anti-inflammatory efficacies. GCBE phytochemicals were analyzed using high-performance liquid chromatography (HPLC). This analysis demonstrated that chlorogenic acid was the predominant component of GCBE, followed by caffeine and caffeic acid. The antioxidant capacity of GCBE was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays, demonstrating significant scavenging capacity with IC₅₀ values of 2.96 ± 1.04 and 7.63 ± 1.03 µg/mL, respectively. The anti-hyperlipidemic efficacy of GCBE was observed through inhibiting cholesterol absorption (by increasing micelle sizes and decreasing cholesterol solubility), lipid digestion, and pancreatic lipase activity *in vitro*. The investigations revealed that GCBE possessed anti-hyperlipidemic properties by inhibiting cholesterol absorption, lipid digestion, and pancreatic lipase activity. Specifically, GCBE increased micelle particle sizes by ~6.5-fold, decreased cholesterol solubility by 2-fold, and reduced pancreatic lipase activity by 25%. Additionally, the *in vitro* anti-hyperglycemic activity of GCBE was evaluated by inhibition of α -amylase and α -glucosidase capacity. GCBE demonstrated anti-hyperglycemic activity by inhibiting α -amylase activity ($32.80 \pm 7.06\%$ inhibition), while α -glucosidase activity remained unaffected. The anti-inflammatory potential of GCBE was evaluated by mRNA regulation using RT-PCR analysis. This analysis revealed that GCBE attenuated mRNA expression of COX-2, TNF- α , IL-1 β , and IL-6 in LPS-induced RAW264.7 cells. GCBE's antioxidant, anti-hyperlipidemic, and anti-hyperglycemic efficacies and its molecular mechanisms in modulating the inflammation pathway found in the present study highlight its potential as a supplement in functional foods or beverages.

Keywords: Coffee; Inflammation; Hyperlipidemia; Hyperglycemia; Diabetes; Functional Food.

1. Introduction

Recently, diabetes prevalence has been increasing across the world. The World Health Organization (WHO) estimates that the number of patients with diabetes will reach 693 million in 2045 [1]. Diabetes is a chronic disease related to carbohydrate metabolism disorder leading to high blood glucose levels, called hyperglycemia. Hyperglycemia is usually

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associated with inflammation, as evidenced by overexpression of proinflammatory cytokines [2]. Prolonged inflammation in hyperglycemia causes damage to numerous tissues, organs, and body systems and, subsequently, to diabetic complications [1]. It has been reported that altering the gastrointestinal tract in obesity with hyperglycemia leads to increased permeability in the intestinal epithelia, tight junctions, and mucosal layer. Subsequently, the leakage of microbial factors, including short-chain fatty acids and lipopolysaccharide (LPS) from the gut microbiota or pathogens, induces inflammation and impairs glucose metabolism [3]. In addition, the production of triglyceride in the gut is controlled by insulin. Type 2 diabetes (T2D) with insulin disorder causes hyperlipidemia. Therefore, consuming foods or drinks containing compounds that inhibit inflammation or improve lipid and carbohydrate metabolism is an alternative way to prevent diabetes.

Besides the carbohydrate metabolism disorder, T2D is also characterized by chronic inflammation through Jun N-terminal kinases (JNKs) and the transcription factor NF-kappa B (NF- κ B), which is related to inflammation and induces insulin resistance [4, 5]. Macrophages, especially RAW264.7 cells, are immune cells widely used as a model to demonstrate the anti-inflammation efficacy of medicinal extracts [6]. Saturated fatty acids, or LPS, induce RAW264.7 cells through the NF-kappa B pathway by binding to toll-like receptor-4 (TLR4). This binding activates the secretion of proinflammatory cytokines (e.g., tumor necrosis factor; TNF- α , interleukin-1 β ; IL-1 β , IL-6) and inflammatory mediators (nitric oxide synthase, iNOS; cyclooxygenase-2, COX-2) [7, 8]. A previous study suggested that increasing cytokines and mediators induce decreased insulin signaling [5]. Many other studies seek to inhibit proinflammatory cytokine secretion using drugs or compounds from medicinal plant extract to increase insulin signaling [3,5,6]. Another study reports on the association between inflammation, hyperglycemia, and diabetes [1,3]. Activation of NF κ B by proinflammatory cytokines in prolonged hyperglycemia results in insulin resistance [9].

Currently, inflammation pathways are a target for diabetes therapy, including anti-diabetic agents and biological anti-inflammatory agents, such as insulin, aspirin, TNF- α antagonists, and IL-1 β antagonists [10]. However, given the increasing prevalence of diabetes, Tsalamandris et al. [10] suggested that research should focus on prevention and treatment. Therefore, functional foods or beverages might provide novel approaches to preventing and controlling T2D.

Across the world, coffee is a popular beverage for adults, with many beneficial effects on health, including reduced incidence of diabetes, metabolic syndrome, and cancer [11, 12]. Prior studies [13–17] have reported that coffee consumption is related to a lower risk of T2D, as observed by blood glucose levels or biomarkers in patients and animal models. Some studies indicate the ability of coffee extract, coffee components (chlorogenic acid, CGA; caffeine, CE; and caffeic acid, CA) [18–21], and green coffee bean extract (GCBE) to improve glucose and lipid metabolism [22–24]. However, the exact cellular mechanisms are not clearly understood, and prior researchers have suggested that the mechanism or pathway of these compounds' effects in T2D should be elucidated.

Therefore, this study aimed to clarify the mechanisms related to decreased T2D. It provides an in vitro study of GCBE to describe its antioxidant, anti-hyperglycemic, anti-diabetic, and anti-inflammatory mechanisms. In the case of anti-inflammation, LPS-induced RAW264.7 macrophage cells were investigated to illustrate the suppression pathway of proinflammatory cytokines and inflammatory mediators using reverse transcription polymerase chain reactions (RT-PCR).

2. Material and Methods

2.1. Material

Folin-Ciocalteu's phenol reagent, dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ABTS, p-nitrophenyl butyrate (p-NPB), 3,5-dinitro salicylic acid (DNS), α -glucosidase, α -amylase, acarbose, phosphatidylcholine, 3-(N-morpholino) propanesulfonic acid (MOPS), cholesterol, sodium taurocholate, ethylenediaminetetraacetic acid (EDTA), 4-nitrophenyl-D-glucopyranoside (pNPG), type II porcine pancreatic lipase, LPS, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). Dulbecco's Modified Eagle's Medium (DMEM), trypsin/EDTA, pen/strep solution, fetal bovine serum (FBS), PureLink RNA Mini Kit, and SuperScript III One-Step PCR with Taq were obtained from Invitrogen, USA. DNA-staining reagent was obtained from Novel Juice, Bio-Helix, Taiwan.

2.2. GCBE Preparation

Green coffee bean materials were ground by using a grinder. Three extractions were performed by boiling green coffee bean powder for 10 min in a ratio of 1:5 (w/v) distilled water. The solution was subsequently freeze-dried using a freeze dryer (CoolSafe 110-4 Pro, LaboGeneTM, Allerød, Denmark) to derive GCBE powder, which was stored at -20°C until use. Analysis of GCBE was performed to investigate its properties following the step in Figure 1.

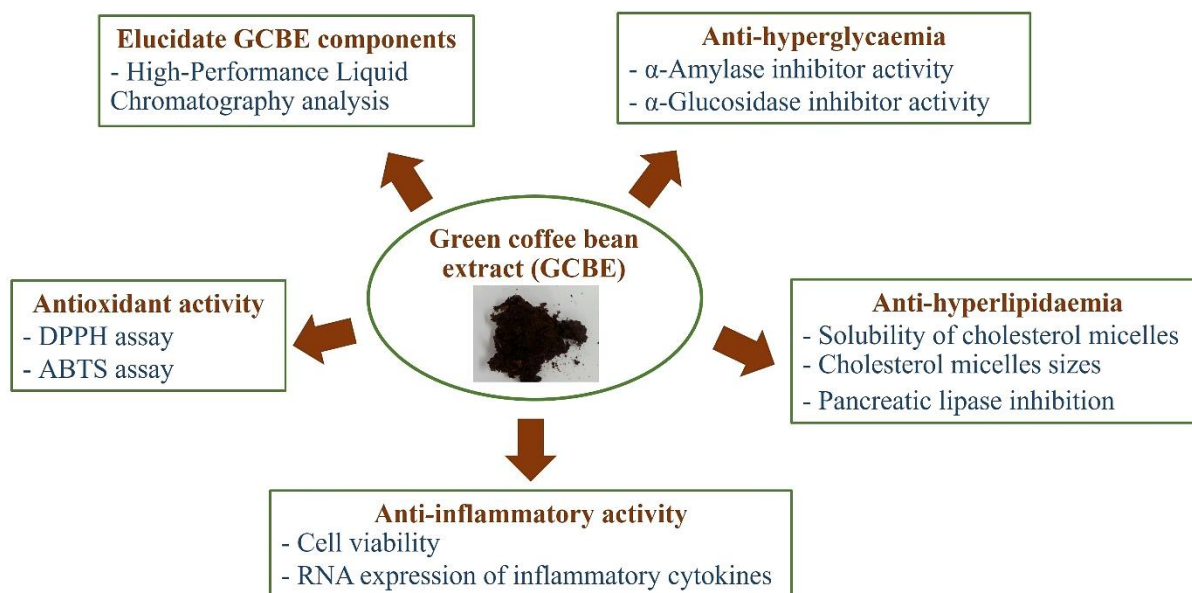


Figure 1. Workflow of the methodology to investigate biological activities of GCBE.

2.3. High-Performance Liquid Chromatography Analysis of GCBE

To determine the levels of the major components, including CGA, CE, and CA, GCBE was run through high-performance liquid chromatography (HPLC). An Agilent 1200 series HPLC instrument (Agilent Technologies, USA) was used for the analysis. Chromatography was run with a 0.5 ml/min flow rate using a C18 column (4.6 x 150 mm, 5 μ m). Formic acid in water (0.1% v/v) was used as a mobile phase A, and formic acid in acetonitrile (0.1% v/v) was used as a mobile phase B. CGA, CE, and CA were detected by UV detection at 280 and 320 nm wavelengths in the chromatogram. GCBE components were measured in milligrams per gram of extract.

2.4. Determination of Antioxidant Activity

2.4.1. DPPH Assay

The antioxidant efficacy of GCBE was investigated by DPPH assay following a previously described procedure [25]. A mixture of GCBE and DPPH was prepared by adding 20 μ L GCBE solution to 180 μ L DPPH in methanol. After incubation at room temperature for 30 min in the dark, the reaction was observed by a microplate spectrophotometer (BioTek, USA) at 517 nm. The antioxidant capacity was presented as IC_{50} compared with the Trolox standard curve.

2.4.2. ABTS Assay

GCBE's scavenging activity was assessed by ABTS assay using the technique of Re et al. [26]. ABTS was conducted in the dark by mixing it with potassium persulphate at room temperature and leaving it for 12–16 hours before use. After adding 100 μ L of GCBE to 1 mL of ABTS, the mixture was incubated for 30 min at ambient temperature in the dark. The scavenging capacity was measured at 734 nm and calculated using Trolox as a standard. All measurements were repeated at least three times. The antioxidant activity was given as IC_{50} .

2.5. In-vitro Anti-hyperlipidemia

2.5.1. Solubility Studies of Cholesterol Micelles

The preparation process for the cholesterol micelle solution was based on an earlier study [27]. The micelle solution was prepared using 1.0 mM sodium taurocholate, 1.0 mM cholesterol, and 0.6 mM phosphatidylcholine. Subsequently, 100 μ g/mL of GCBE was added to the micelle solutions to solute the cholesterol micelles for 3 h. A 0.22 μ m filter membrane was used to filter the mixed solution. The filtrate solution was investigated using cholesterol assay kits. The cholesterol micellar solubility was presented as the cholesterol concentration in mg/dL.

2.5.2. Determination of Cholesterol Micelles Sizes

Slight adjustments were made to the prior methodology [27, 28] to determine the increased cholesterol micelles sizes using GCBE. The cholesterol micellar solutions were developed using sodium taurocholate, phosphatidylcholine, and cholesterol. Subsequently, the cholesterol micellar solutions were exposed to 100 μ g/mL GCBE. After 3 h of incubation at 37°C, the sizes of the cholesterol micelles were measured by analyzing the solution with a particle size analyzer.

2.5.3. Pancreatic Lipase Inhibition Assays

Pancreatic lipase was used as an enzyme model to investigate GCBE's inhibitory capacity following a previous study with some modifications [29]. p-NPB was used as a substrate to investigate lipase activity. Porcine pancreatic lipase was dissolved in a buffer to derive an enzyme solution. Buffer was prepared by mixing EDTA (1 mM) and MOPS (10 mM) in Tris-HCL (100 mM) supplemented with CaCl₂ (5 mM). The enzyme solution was mixed with 2.5 mg/mL GCBE and incubated for 15 min at 37°C to derive the reaction. Subsequently, 10 mM p-NPB in dimethyl formamide was mixed into the reaction for 30 min at 37°C. To identify the end-product (p-nitrophenol), a microplate spectrophotometer (BioTek, USA) was used to monitor the reaction at 405 nm. The amount of enzyme activity inhibition was compared to the control and calculated by the following formula:

$$\% \text{ inhibition} = \frac{[(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}) \times 100]}{\text{Absorbance}_{\text{control}}}$$

2.6. In-vitro Anti-hyperglycemia

2.6.1. α -Amylase Inhibitor Activity

The α -amylase inhibition efficacy of GCBE was determined following methods used previously [30]. α -amylase was pre-incubated with 5 mg/mL GCBE for 10 min at 37°C. Starch solution (2 mg/mL) was used as a substrate for this reaction. After incubation at 37°C for 20 min, the reaction was terminated by adding 1% DNS and boiling for 5 min. The reaction was observed at the absorbance of 540 nm compared with the reaction of 200 μ M acarbose, used as a positive control. Inhibition of α -amylase activity was presented as a percentage of acarbose.

2.6.2. α -Glucosidase Inhibitor Activity

The method of α -glucosidase inhibition activity assay was performed according to a previous study [30]. α -glucosidase was prepared in a potassium phosphate buffer (0.1 M) and pre-incubated with GCBE (5 mg/mL) for 15 min at 37°C after incubation with 400 μ M pNPG at 37°C for 40 min. The reaction was terminated by 0.1 M Na₂CO₃, and the activity was observed at 405 nm. Inhibition of α -glucosidase activity was calculated and presented as a percent inhibition compared with 2mM acarbose as a positive control.

2.7. Anti-inflammatory Activity of GCBE on LPS-induced RAW264.7 Cells

2.7.1. Determination of Cell Viability

To check that the downregulation of mRNA is not the result of cell death, the viability of cells treated with GCBE at various concentrations was determined. Consequently, the optimum concentrations were used for further investigations.

RAW264.7 cell lines (ATCC[®], TIB-71[™], USA) were used as the model for this study. They were cultured in DMEM containing 10% FBS and 1% pen/strep at 37°C with 5% CO₂. Cell viability was examined in a 96-well culture plate by seeding RAW264.7 cells (5 x 10³ cells) in each well. Fresh serum-free DMEM containing GCBE at various concentrations (12.5–200 μ g/mL) was replaced after 24 h incubation. The experiment was divided into three groups: (i) DMEM for 24 h (control), (ii) 1 μ g/mL LPS for 24 h, (iii) 12.5–200 μ g/mL GCBE for 24 h and followed by 1 μ g/mL LPS for 24 h. The experiments were mixed with 50 μ L MTT and further incubated for 4 h. Finally, the formazan crystals were dissolved by 100 μ L DMSO, and the reaction was observed at the absorbance of 590 nm using a microplate reader (BioTek, USA). The results were calculated, compared with the control group, and presented as the percent cell viability.

2.7.2. Total RNA Extraction and Investigation of RNA Expression by RT-PCR

The experiment was performed in a 6-well culture plate by culturing RAW 264.7 cells (1.5 x 10⁵ cells/well) in DMEM containing 10% FBS and 1% pen/strep for 24 h. The treatment was designed as three groups, the same as for the determination of cell viability (section 2.7.1). The expression of mRNA transcript to proinflammatory cytokines (COX-2, iNOS) and inflammation mediators (TNF- α , IL-1 β , and IL-6) was used to evaluate the inflammatory inhibition efficacy of GCBE, following previous methods [31] with some modifications. After treatment in each group, a Purelink RNA mini-Kit was used for mRNA extraction, and the RNA concentration was measured using the NanoDrop Lite spectrophotometer (Thermo Scientific, USA). The extracted RNA was then converted to cDNA and amplified using superscript III one-step PCR with Tag in a thermal cycle (Veriti 96-well fast thermal cycle, Thermo Fisher, Singapore). The specified nucleotide primers included:

COX-2 (F-5'CACTACATCCTGACCCACTT3', R-5'ATGCTCCTGCTTGAGTATGT 3'),

iNOS (F-5'CCCTTCCGAAGTTTCTGGCAGCAGC3', R-5'GGCTGTCAGAGCCTCGTGGCTTTGG3'),

TNF- α (F-5'TCTCATCAGTTCTATGGCCC3', R-5'GGGAGTAGACAAGGTACAAC3'),

IL-1 β (F-5'TGGACGGACCCCAAAAGATG3', R-5'AGAAGGTGCTCATGTCCTCA3'), and

IL-6 (F-5'GTTCTCTGGGAAATCGTGA3', R-5'TGTACTCCAGGTAGCTATGG3').

The PCR reaction was performed by 40 cycles of 94°C denaturation (30 s), 55°C annealing (30 s), and 72°C extension (45 s). Gel electrophoresis on a 1.2% agarose gel containing a DNA-staining reagent was used to observe the PCR products. The RNA expression was quantified by imaging the gel using Gel Doc XR+ (BIO-RAD, California, USA) and densitometric analysis using image la software (Gel Doc XR+, BIO-RAD, California, USA).

2.8. Statistical Analysis

The results of the anti-hyperglycemia and anti-hyperlipidemia assays were presented as means \pm standard error (SE), while other results were presented as means \pm standard deviation (SD) of three independent replications. Statistical significance was analyzed using one-way analysis of variance (ANOVA), and pairwise comparisons were made with the control group using Dunnett's t-test (with a threshold value of $p < 0.05$). Statistical analyses were performed using IBM SPSS Statistics 29.

3. Results

3.1. Phytochemical and Antioxidant Efficacy of GCBE

The HPLC chromatogram of GCBE is shown in Table 1. It indicates that CGA was the main compound with a concentration of 294.1 mg/g, followed by CE and CA with concentrations of 58.6 and 27.2 mg/g, respectively. The antioxidant capacity of GCBE is shown in Table 2. DPPH assays demonstrated that GCBE effectively reduced free radicals better than Trolox ($IC_{50} = 2.96 \pm 1.04 \mu\text{g/mL}$, compared with $4.57 \pm 1.07 \mu\text{g/mL}$). The antioxidant capacity, measured by ABTS assay, indicated an IC_{50} at $7.63 \pm 1.03 \text{ mg/mL}$ for GCBE, compared with $3.61 \pm 1.44 \text{ mg/mL}$ for Trolox.

Table 1. Quantification of main polyphenols contained in GCBE by HPLC

Phenolic compounds	Concentration (mg/g extract)
Chlorogenic acid	294.1
Caffeine	58.6
Caffeic acid	27.2

Table 2. Antioxidant capacity of GCBE by DPPH and ABTS assay

Samples	DPPH radical scavenging activity IC_{50} ($\mu\text{g/mL}$)	ABTS radical scavenging activity IC_{50} (mg/mL)
green coffee bean extract	2.96 ± 1.04	7.63 ± 1.03
Trolox	4.57 ± 1.07	3.61 ± 1.44

3.2. Effect of GCBE on Anti-hyperlipidemic Activity

This study evaluated GCBE's anti-hyperlipidemic effect using cholesterol micelle formation size and solubility, as well as pancreatic lipase inhibition. The size of cholesterol micelles showed that GCBE treatment effectively increased the particle size of micelles compared with the control. The mean size of control cholesterol-mixed micelles was $124.98 \pm 12.8 \text{ nm}$, whereas the mean particle size with GCBE was $805.88 \pm 140.72 \text{ nm}$. The larger micelle size with GCBE means absorption is more difficult than that of the control, by ~ 6.5 times. The result of cholesterol micelle solubility revealed the GCBE suppression effect on cholesterol solubility. Treatment with GCBE decreased the solubility to $24.30 \pm 2.39 \text{ mg/dL}$, whereas that of the control was $50 \pm 0 \text{ mg/dL}$. It decreased to two times compared with the control, which indicated that lower solubility causes reduced digestion. Finally, the pancreatic lipase inhibition by 2.5 mg/mL GCBE was presented as $24.73 \pm 2.86\%$, while that of the positive control was $75.57 \pm 0.73\%$. GCBE could inhibit pancreatic lipase activity, even if it does so less than the control. These data presented the anti-hyperlipidemic properties of GCBE by inhibiting cholesterol absorption, lipid digestion, and pancreatic lipase activity.

3.3. Effect of GCBE on Anti-hyperglycemic Activity

The anti-hyperglycemic capacity of GCBE was evaluated by inhibiting α -amylase and α -glucosidase activity. The results showed that GCBE could effectively inhibit the activity of α -amylase by presenting the inhibition at $32.80 \pm 7.06\%$, even though the inhibition by acarbose (positive control) was presented as $85.33 \pm 12.02\%$. This inhibition of α -amylase activity indicates that GCBE decreases carbohydrate digestion. However, the GCBE could not inhibit α -glucosidase activity, whereas the positive control presented as $88.05 \pm 16.42\%$ inhibition.

3.4. Effect of GCBE on RAW264.7 Cell Viability

The effect of GCBE and the related compounds (CGA, CE, and CA) on the non-LPS induced RAW264.7 cells was observed by cell viability after treatment with the extract or the compounds at concentrations of 100–500 µg/mL. The results showed that cells were unaffected by the extract and all compounds at 100–200 µg/mL concentrations. However, the viability of cells treated with GCBE, CGA, and CA was reduced at higher concentrations (300–500 µg/mL), as shown in Figure 2. According to the correlation results of cell viability, the concentrations for anti-inflammation activity investigation were not more than 200 µg/mL.

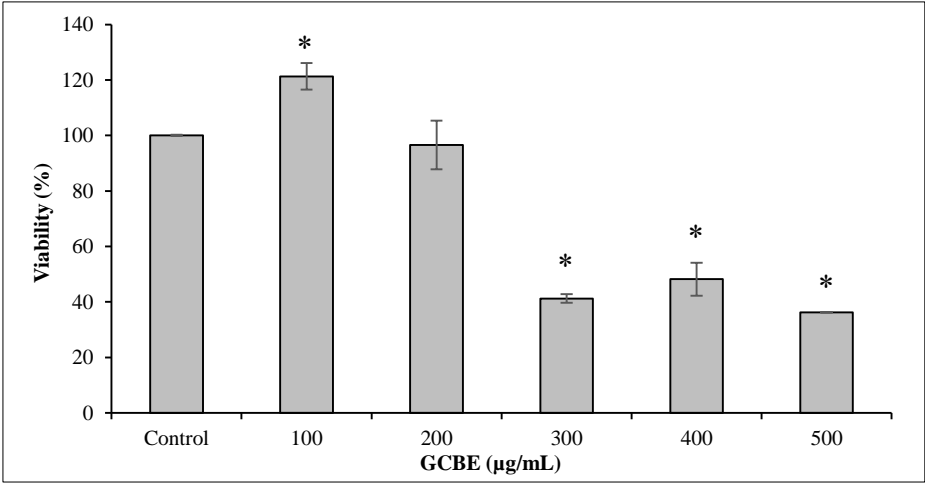


Figure 2. Cell viability of RAW264.7 cells after treatment with various concentrations of GCBE compared with control (n=3). **p* < 0.05

3.5. Effect of GCBE and Related Main Compounds on iNOS and COX-2 mRNA Expression

The secretion of COX-2 and iNOS mRNA from LPS-induced RAW264.7 cells was investigated to evaluate the anti-inflammatory properties of GCBE compared with the related main compounds. Figure 3a shows the mRNA expression from RAW264.7 cells exposed to various concentrations of GCBE and each pure compound (CGA, CE, and CA). The result shows that GCBE, CGA, CE, and CA could effectively reduce COX-2 expression. The quantitative mRNA expression is presented in Figure 3b. It indicates the downregulation of COX-2 resulting from GCBE, CGA, CE, and CA in a dose-dependent manner. The result of iNOS mRNA expression observed by visualization showed that, after treatment with various concentrations of GCBE, CGA, CE, and CA was not different (Figure 3c). However, the quantitative expression of iNOS mRNA in Figure 3d indicated the reduction of expression in cells treated with CGA and CE in a dose-dependent manner, while it was not reduced in cells treated with GCBE and CA.

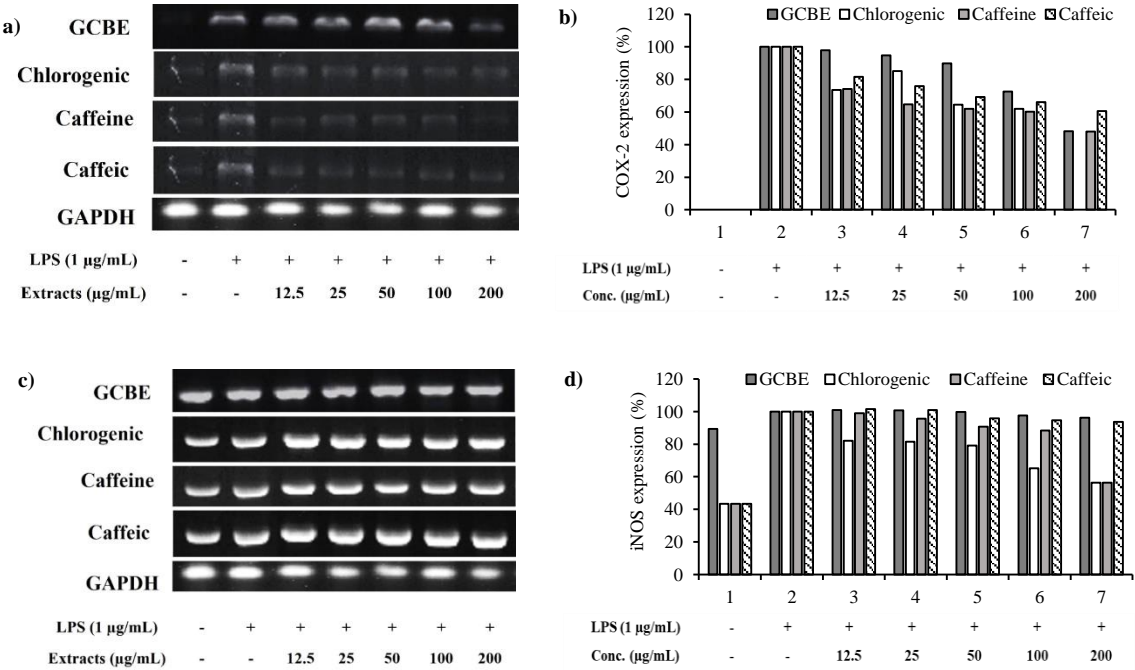


Figure 3. Effect of GCBE on inflammatory mediator production in LPS-induced RAW264.7 cells. (a) Expression of inducible nitric oxide synthase (iNOS) mRNA and (b) densitometry expression levels. (c) Expression of cyclooxygenase 2 (COX-2) mRNA and (d) densitometry expression levels.

3.6. Effect of GCBE and Related Main Compounds on TNF- α , IL-1 β , and IL-6 mRNA Expression

To determine the inflammatory suppression ability of GCBE, expressions of TNF- α , IL-1 β , and IL-6 mRNA were investigated using RT-PCR. The TNF- α mRNA expression after cell treatment with GCBE, CGA, CE, and CA by electrophoresis is presented in Figure 4. The TNF- α expression in Figure 4a indicated decreased expression when treated with GCBE, CGA, CE, and CA in a dose-dependent manner. Accordingly, related band densitometry (Figure 4b) showed that TNF- α expression in cells treated with GCBE was significantly reduced. Remarkably, at a concentration of 100 $\mu\text{g/mL}$ GCBE treatment, TNF- α expression was reduced to 45.78%, and it completely disappeared at 200 $\mu\text{g/mL}$ GCBE treatment. Treatment with CGA, CE, and CA showed an effect in reducing TNF- α expression, except for CA at concentrations of 12.5 and 25 $\mu\text{g/mL}$ (Figure 4b). Figures 5a and 5b show the expression of IL-1 β and its expression level after treatment with the extract or related main compounds. It was observed that IL-1 β expression was slightly reduced when treated with GCBE, CGA, and CE, while CA did not significantly reduce IL-1 β expression. The expression of IL-6 showed a notable and significant decrease upon treatment with GCBE, CGA, CE, and CA in a dose-dependent pattern (Figures 6a and 6b).

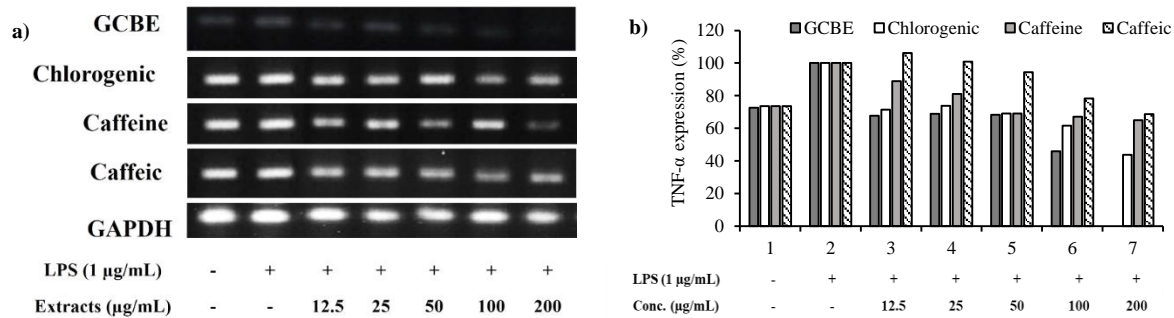


Figure 4. Effect of GCBE on the expression of tumor necrosis factor-alpha (TNF- α) mRNA from LPS-induced RAW264.7 cells (a) and densitometry expression levels (b)

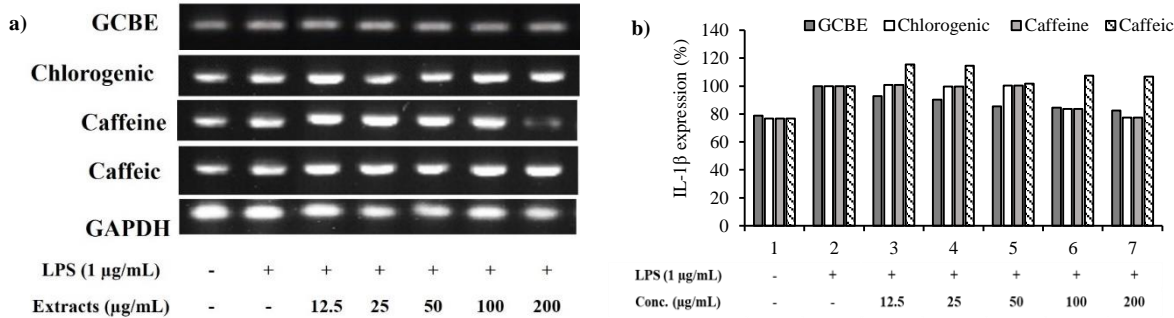


Figure 5. Effect of GCBE on the expression of interleukin-1 β (IL-1 β) mRNA from LPS-induced RAW264.7 cells (a) and densitometry expression levels (b)

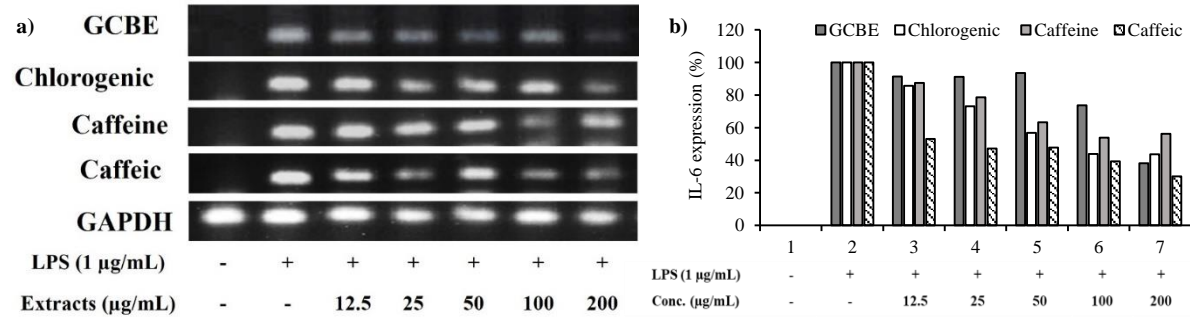


Figure 6. Effect of GCBE on the expression of interleukin-6 (IL-6) mRNA from LPS-induced RAW264.7 cells (a) and densitometry expression levels (b)

4. Discussion

Recently, it has been reported that coffee consumption is associated with the prevention of diabetes. However, the mechanism of this effect is not clearly understood. The results of the present study clarified the mechanism of T2D prevention by GCBE in pathways related to lipid and carbohydrate metabolism and anti-inflammation. It was found that

GCBE contained rich phenolic acids, particularly CGA and its derivative compounds. The quantitative HPLC results revealed a significant amount of CGA (up to 294.1 mg/g GCBE extract), followed by CE (58.6 mg/g) and CA (27.2 mg/g). These results are consistent with previous studies that reported abundant CGA in coffee and green coffee beans [12, 18, 19, 21].

Furthermore, our results confirmed previous reports that GCBE strongly affected antioxidant activity in both the DPPH and ABTS assay, similar to Trolox. We assume that this effect was attributed to the phenolic compounds, especially CGA, CE, and CA. CGA demonstrates a broad spectrum of potential health benefits, including antioxidant, antibacterial, anti-inflammatory, and anti-carcinogenic effects [12, 18, 21, 32].

Our results demonstrate that GCBE possesses anti-hyperlipidemic properties by inhibiting cholesterol absorption, lipid digestion, and pancreatic lipase activity. Intestinal lipid absorption involves cholesterol-mixed micelles. Forming cholesterol-mixed micelles affects absorption enhancement [30, 33]. This study indicated that GCBE could inhibit lipid absorption by increasing cholesterol-mixed micelle size and reducing lipid solubility. GCBE induced a ~6.5-fold increase in cholesterol micelle size and a 2-fold reduction in cholesterol solubility, making cholesterol absorption more difficult. It probably implies that GCBE consumption utilizes the increasing micelle size and decreasing lipid solubility, leading to decreased lipid absorption in the intestine. As pancreatic lipase plays a key role in lipid digestion, inhibiting its activity is another mechanism for reducing hyperlipidemia. GCBE could suppress this enzyme by approximately 25%. Thus, inhibiting pancreatic lipase activity is one strategy to reduce lipid absorption and lipid blood levels. This result is consistent with a previous study that reported the lipid-preventing capacity of green coffee beans in humans [23, 24]. Decreasing coffee's cholesterol solubility was attributed to its soluble fibers and lipid content, breaking bile salt down from micelle solution and forming a larger size [34].

Carbohydrate digestion and glucose uptake are associated with α -amylase and α -glucosidase activity, which regulate blood sugar levels [35]. Increased blood sugar levels (hyperglycemia) can activate IL-1 β secretion. High IL-1 β secretion subsequently induces inflammation of pancreatic beta cells [36]. In the case of T2D, this presents an imbalance between glucose absorption and insulin secretion. Insufficient insulin secretion cannot regulate blood sugar levels and results in hyperglycemia [9, 35]. A potential therapeutic approach to overcome hyperglycemia is inhibiting starch digestion of enzymes such as α -amylase and α -glucosidase. This study indicates that GCBE could inhibit α -amylase activity by 32.8% even though α -glucosidase activity was unaffected. The inhibition of α -amylase activity by GCBE was attributed to its phenolic components binding to the enzyme and forming a complex [35]. The digestive enzyme binding with GCBE is inactivated, reducing blood sugar levels. Therefore, the present results indicate that GCBE is a potential compound to act against hyperglycemia, consistent with results reported in previous studies *in vitro*, *in vivo*, and in clinical trials [13, 35, 37].

Macrophages are usually used as a model to study the anti-inflammatory effect of drugs or plant extract because they are the principal immune cells responsible for inflammation of various organs. Macrophages produce cytokines and reactive oxygen species (ROS), producing oxidative stress and increased activation of the inflammation pathway [38]. LPS activates macrophages to produce inflammation mediators COX-2 and iNOS, linked to diseases such as Alzheimer's, cancer, and diabetes [6]. Our results indicate that GCBE and its components could reduce COX-2 mRNA expression. mRNA expression of iNOS was reduced by the effect of CGA and CE, whereas GCBE and CA were unaffected. Although GCBE did not affect iNOS expression, it indicated a suppressive effect on COX-2. iNOS expression leads to NO production and regulates COX-2 expression [19]. Therefore, inhibition of the COX-2 pathway is a form of inflammation therapy. GCBE's COX-2 inhibition effect was attributed to its phenolic components (CGA, CE, and CA), as shown in the results of its pure components.

In addition, GCBE has been shown to suppress the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 expression, the same as CGA and CE. In the case of CA, inhibition of TNF- α and IL-6 expression was indicated, while the expression of IL-1 β did not occur. Accordingly, GCBE was remarkable in its anti-inflammatory effect, inhibiting inflammatory mediators and proinflammatory cytokines. The anti-inflammatory efficacy shown in the present study agreed with a previous study of GCBE in rats [39] and of the extracts from coffee leaves and coffee pulp in RAW264.7 cells [19]. These reactions are attributed to the phenolic content in GCBE and coffee extracts, including CGA, CA, and CE [21]. The phenolic compounds likely inhibited the TLR4 receptor or NF κ B signaling pathway associated with COX-2, TNF- α , and IL-6 production [5].

Many cytokines associated with organ inflammation related to diabetes—such as TNF- α , IL-1 β , and IL-6—are usually triggered through the transcription factor NF κ B macrophage pathway. These inflammation molecules affect COX-2 and iNOS upregulation [3]. The association of inflammation with obesity and diabetes by the occurrence of mediators and proinflammatory cytokines in blood circulation has been reported [38]. Accordingly, anti-inflammatory drugs and natural compounds from plants may be used to reduce inflammation and improve glycemia in T2D patients [3]. An interesting strategy for diabetes prevention and control is aiming to suppress several inflammation response pathways rather than focusing on one pathway. Adopting an anti-inflammatory diet is a novel approach to preventing and controlling T2D [10]. Therefore, there is interest in GCBE as a functional food or beverage due to its anti-inflammatory effects on suppressing inflammation responses related to diabetes.

5. Conclusion

The present results indicate that GCBE effectively reduces hyperglycemia by inhibiting α -amylase activity and reducing hyperlipidemia by interrupting lipid digestion and absorption. Moreover, GCBE has a high potential antioxidant capacity and indicated anti-inflammatory activity to prevent inflammation by blocking the expression of COX-2, TNF- α , and IL-6 mRNA expression. The results from the present study provide information on the benefits and the molecular mechanisms behind GCBE's inflammation pathway. With further study, GCBE shows potential for development as a functional food or beverage.

6. Declarations

6.1. Author Contributions

Conceptualization, A.D. and A.Y.; methodology, A.R., A.S., and A.K.; software, N.Y.; validation, A.D., N.Y., and Y.Y.; writing—original draft preparation, A.D. and A.R.; writing—review and editing, A.D. and A.Y.; project administration, A.D. and A.Y.; funding acquisition, A.D. and A.Y. All authors have read and agreed to the published version of the manuscript.

6.2. Data Availability Statement

The data presented in this study are available in the article.

6.3. Funding

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6.5. Institutional Review Board Statement

Not applicable.

6.6. Informed Consent Statement

Not applicable.

6.7. Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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