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Original Research Article

Ameliorative effect of *Citrus aurantifolia* and *Cinnamomum burmannii* extracts on diabetic complications in a hyperglycemic rat model

Herin Mawarti*, Khotimah, Mohammad Zulfikar As'ad, Mukhammad Rajin
Department of Nursing Sciences, Faculty of Health Sciences, Pesantren Tinggi Darul Ulum University, Jombang, East Java 61481, Indonesia

For correspondence:* ***Email:* *herinmawarti@fik.unipdu.ac.id;* ***Tel:*** *+62-85736347827*

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Abstract

Purpose: *To evaluate the effects of Citrus aurantifolia and Cinnamomum burmannii extracts (Jermanis) on the number of pancreatic β-cells and levels of superoxide dismutase (SOD), low-density lipoprotein (LDL), and transforming growth factor-beta (TGF-β) in β-cells.*
Methods: *Various doses of a combination of extracts of C. aurantifolia and C. burmannii were given orally to 25 male Wistar rats (Rattus norvegicus) every 2 h for 30 days. Doses of 100, 300 and 500 mg/kg/day C. aurantifolia extract were considered low, medium and high doses, respectively, while, 200, 400 and 800 mg/kg/day C. burmannii extract were considered low, medium and high doses, respectively. LDL and SOD levels in blood serum were analyzed spectrophotometrically. TGF-β expression was evaluated by immunohistochemistry. Pancreatic tissue sections (diameter of a β-cell) were evaluated by light microscopy after hematoxylin and eosin staining.*
Results: *The combination of C. aurantifolia and C. burmannii extracts increased SOD levels, TGF-β expression and the number of β-cells and decreased LDL levels in hyperglycemic rats. The results indicate that the highest doses of C. aurantifolia and C. burmannii increased the number of β-cells in the islets of Langerhans. The combined extracts of C. aurantifolia and C. burmannii significantly affected pancreatic cell regeneration.*
Conclusion: *The combination of C. aurantifolia and C. burmannii extracts may be a promising alternative preventative medicine for management of diabetic complications in patients with hyperglycemia.*

Keywords: *β-cell, Diabetes, Diabetic complications, Citrus aurantifolia, Cinnamomum burmannii, Biochemical profile, TGF-β expression*

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INTRODUCTION

Diabetes mellitus (DM) is a chronic disease that is increasing globally, including Indonesia. DM is related to economic status and lifestyle changes [1]. The prevalence of DM in urban regions is approximately 5.7 % [2,3]. In the United Kingdom, DM (all types) is estimated to affect approximately 2.4 million people (prevalence of 4 %), of which 1.4 million (prevalence of 2.5 %) are

diagnosed, while 1 million go undiagnosed [4].

If DM is not well controlled, it leads to NAD(P)H oxidation, which induces the production of free radicals [5]. Excessive production of free radicals can trigger oxidative stress, which can damage lipids and thereby increase the level of low-density lipoprotein (LDL) in the blood [6]. Free radicals can damage the smooth muscle cells of blood vessels, which are a source of superoxide dismutase (SOD) [7].

As the effects of free radicals in DM can lead to complications, additional antioxidants are required. *Citrus aurantifolia* and *Cinnamomum burmannii* contain flavonoids with antioxidant properties and have a strong protective effect against radiation exposure [8,9]. One of the growth factors involved in pancreatic organogenesis is transforming growth factor-beta (TGF-β) [10-12].

In this study, hyperglycemic rats were fed a high-cholesterol diet and treated with a combination of extracts from *C. aurantifolia* and *C. burmannii*, which have antioxidant activity. The effect of the extract treatment on the complications caused by hyperglycemia and the high-cholesterol diet were assessed. A previous study showed that flavonoid antioxidants derived from green tea decreased cholesterol, glucose and triglyceride levels in rats on a high-fat diet [13]. Therefore, the aim of this study was to evaluate the effect of a combination *C. aurantifolia* and *C. burmannii* extracts on SOD and LDL levels, TGF-β expression and the number of β-cells in the islets of Langerhans in a hyperglycemic rat model. Our results showed that a combination of *C. aurantifolia* and *C. burmannii* extracts is a promising therapeutic medicine to prevent diabetic complications.

EXPERIMENTAL

Preparation of rats

Twenty-five male rats (*Rattus novergicus*) were acclimatized under laboratory conditions for 1 week with adequate feeding. On the last day, fasting blood glucose levels were measured, and the rats with normal blood sugar levels (90 – 110 mg/dl) were selected. The rats were intraperitoneally injected with streptozotocin at 30 mg/kg body weight (BW). A week after streptozotocin induction, blood glucose levels were measured after fasting for 6 hours. Rats with a sugar level > 250 mg/dl (using a blood glucose meter) were considered hyperglycemic. The Ethics Committee of Polytechnic of Health, The Ministry of Health in Malang approved this study (approval ref no. 100/KEPK-POLKESMA/2015). The experimental procedures were performed according to the principles of the Institute for Laboratory Animal Research in the *Guide for the Care and Use of Laboratory Animals* [14].

Preparation of *Citrus aurantifolia* and *Cinnamomum burmannii* extracts

C. aurantifolia juice was filtered using flannelette and then dried using a freeze dryer. *C. burmannii* was powdered using size 40 mesh. Then, 1 kg *C. burmannii* powder was dissolved in 7 l 95% ethanol, with occasional stirring for 24 hours, and then filtered. The filtrate was collected, and the precipitate was macerated for 24 hours, and re-maceration was conducted for 2–5 days. The collected filtrate was evaporated at 60˚C until it turned into a thick liquid. This thick liquid was considered the extract.

Rat treatment

Rats were divided into five treatment groups: (1) negative control, standard diet (G1); (2) positive control, high-cholesterol diet (G2); (3) high-cholesterol diet + *C. aurantifolia* 100 mg/kg/day and *C. burmannii* 200 mg/kg/day (G3); (4) high-cholesterol diet + *C. aurantifolia* 300 mg/kg/day and *C. burmannii* 400 mg/kg/day (G4); and (5) high-cholesterol diet + *C. aurantifolia* 500 mg/kg/day and *C. burmannii* 800 mg/kg/day (G5).

Prior to treatment, rats were adapted to laboratory conditions for 7 days to adjust to the environment (e.g., cage changes and feeding times) and were fed a standard feed diet during this adaptation period.

In groups 3, 4 and 5, a few days after alloxan induction, the rat's blood glucose levels were examined immediately because the extract was given when the blood glucose level was above 200 mg/dl (5–6 days). The treatment was carried out over the course 30 days, then the glucose level, LDL, and SOD were examined.

Examination of fasting blood serum LDL (low-density lipoprotein) level

The blood (~ 3 mL) was collected from rats' heart and then centrifuged at 3000 rpm for 20 minutes. Then serum was collected and analyzed. LDL measurements were conducted using specific colorimetric tests (Horiba ABX Diagnostics, Montpellier, France) and an automated analysis system (COBAS MIRA, Roche, Basel, Switzerland). LDL levels were then measured

using ABX PENTRA CP (ABX Pentra, Montpellier, France).

Measurement of superoxide dismutase content

The blood (~ 3 mL) was centrifuged at 3500 rpm for 20 min. The supernatant was collected and centrifuged at 6000 rpm for 10 minutes at 4˚C and then transferred to Eppendorf tubes. The supernatant was diluted with xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), and phosphate-buffered saline (PBS). The sample was diluted with PBS to a total volume of 3500 µL and incubated at 30°C for 30 minutes (until the sample turned purple). The SOD level was measured by UV/Vis spectrophotometry (500 – 600 nm).

Preparation of pancreatic sections for histological examination

The pancreas was solidified in a paraffin block and then cut into 5 µm sections using a microtome. Sections were dried using a hot plate at 38 – 40 °C and then stored in an incubator at 38 – 40 °C for 24 h.

Evaluation of transforming growth factor-beta expression

TGF-β expression was determined using immunohistochemical methods. Prepared slides were washed with PBS (pH 7.4) and then 3 % Hydrogen Peroxide (H_2O_2) for 20 min. The slides were washed again with PBS (pH 7.4) for 5 minutes (three times). The slides were blocked with 5 % fetal bovine serum for 1 hour and then washed with PBS (pH 7.4) for 5 min (three times). The prepared slides were incubated with primary anti-rat TGF-β antibody [antibodies-online GmbH, Schloss-Rahe-Str. 15, 52072 Aachen, Germany] overnight at 4 °C and then washed with PBS (pH 7.4) for 5 min (three times).

The slides were incubated with a secondary anti-biotin antibody (Santa Cruz Biotechnology, USA) for 1 h at room temperature, washed with PBS (pH 7.4) for 5 min (three times) and incubated with streptavidin-horseradish peroxidase for 40 min. The slides were washed with PBS (pH 7.4) for 5 minutes (three washes) and then incubated for 10 minutes with diaminobenzidine (DAB).

The slides were washed with PBS (pH 7.4) for 5 min (three washes). Counterstaining was performed using Mayer's hematoxylin for 10 min. The slides were washed and dried in Entellan mounting medium and covered with a cover glass. Positive TGF-β expression was indicated by a brown staining color. The percentage of cells positive for TGF-β expression was calculated using Axiovision software.

Pancreatic tissue repair evaluation using hematoxylin and eosin staining

For hematoxylin and eosin staining, tissues were first deparaffinized and then placed in storied xylol for 5 minutes. During the rehydration stage, the prepared tissues were placed in a graded ethanol series (95, 90, 80 and 70 %) for 5 min and then soaked in distilled water for 5 min. Hematoxylin was added for 10 minutes until the sections showed strong staining. The tissues were washed with water for 30 min and then rinsed with distilled water before staining with eosin for 5 min. Then, dehydration was performed by subjecting the tissues to a series of ethanol washes consisting of 80, 90, 95 % and absolute ethanol. The tissues were cleared by placing in 1,2 xylol and were then dried and mounted with Entellan.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 1.9. The data were assessed for normal distribution and variance, and differences between groups were determined by one-way ANOVA with Tukey's test. Differences between groups were considered significant at $p < 0.05$.

RESULTS

The results showed that the extracts of *C. aurantifolia* and *C. burmannii* had a significant effect on blood glucose levels ($p = 0.035$) (Figure 1). There was no difference between the negative control group (G1) and the G4 group that consumed *C. aurantifolia* (300 mg/kg/day) and *C. burmannii* (400 mg/kg/day). The *C. aurantifolia* and *C. burmannii* extracts lowered the glucose level by 53.2 %. The decrease in glucose levels in the other groups were not significant.

The *C. aurantifolia* and *C. burmannii* extracts also had a significant effect on SOD levels in the blood ($p = 0.00$). There was a significant difference between the negative control group (K1) and the groups K3, K4 and K5. However, there was no significant difference between the groups that were treated with the extracts of *C. aurantifolia* and *C. burmannii*. The results showed that the highest increase in SOD level occurred when the dose of *C. aurantifolia* was 300 mg/kg/day and the dose of *C. burmannii* was

400 mg/kg/day. The extracts of *C. aurantifolia* and *C. burmannii* was able to increase the SOD level by 75.86 % in group K4, 73.4 % in group K3 and 66.9 % in group K5 (Figure 2).

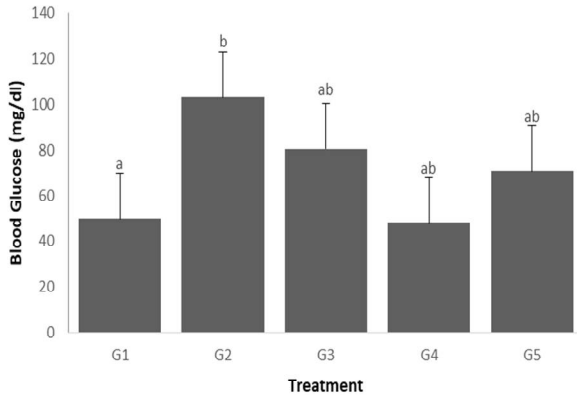

Figure 1: Effect of the extract of *C. aurantifolia* and *C. burmannii* on blood glucose levels in fasting Wistar rats after treatment.

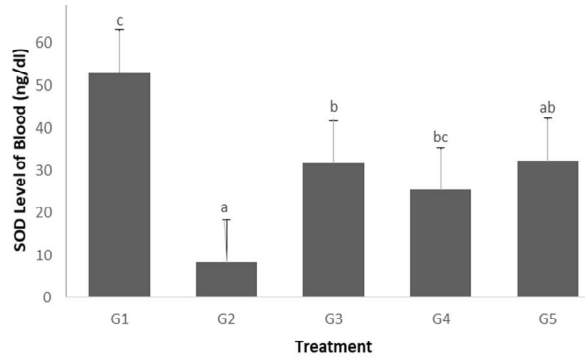

Figure 2 Effect of the combined extract of *C. aurantifolia* and *C. burmannii* on SOD levels (mg/mL) in fasting Wistar rats after treatment

The LDL levels (Figure 3) showed that the extracts of *C. aurantifolia* and *C. burmannii* had a significant effect on cholesterol levels (p = 0.00). There were significant differences among the negative control (G1), G2, G3, G4 and G5 groups, with the lowest LDL levels found in the G1 and G5 groups (treated with 500 and 800 mg/kg/day *C. aurantifolia* and *C. burmannii* extracts, respectively).

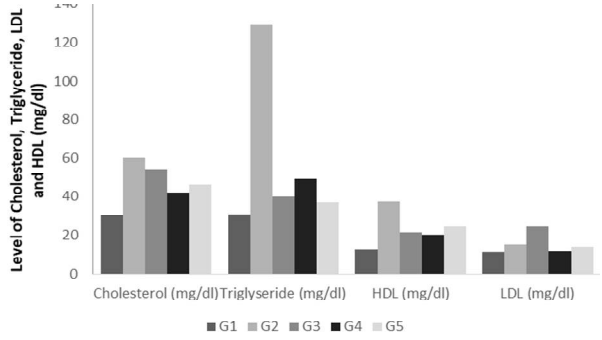

Figure 3: Low-density lipoprotein blood levels (mg/dl) in fasting Wistar rats after treatment

Neogenesis was analyzed by quantifying the number of β-cells. TGF-β levels and the number of β-cells in the islets of Langerhans were correlated with the dose of *C. aurantifolia* and *C. burmannii* extracts. The highest doses of the extracts increased the number of β-cells significantly (Figure 4a). In addition, to confirm the effect of the extract, TGF-β expression was evaluated as an indicator of improvement. The results showed that treatment with the *C. aurantifolia* and *C. burmannii* extracts increased the expression of TGF-β (Figure 4b). β-Cell death in diabetic patients triggers the expression of several cytokines, such as IL-1β, TNF-α, IFN-β and IFN-γ. Administration of the *C. aurantifolia* and *C. burmannii* extracts prevented β-cell death. β-cell regeneration is a self-renewal process that requires TGF-β expression [15].

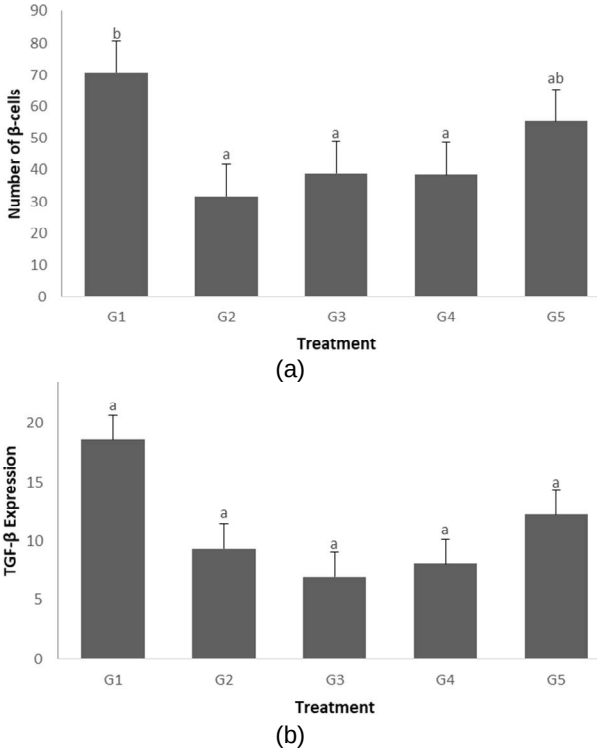

Figure 4: Effect of the extract of *C. aurantifolia* and *C. burmannii* on the number of β-cells (a) and TGF-β expression (b)

DISCUSSION

The results showed that extracts of *C. aurantifolia* and *C. burmannii* had a significant effect on blood glucose and SOD levels. The extracts lowered the glucose level by 53.2%, and the 300 mg/kg BW/day *C. aurantifolia* and 400 mg/kg BW/day *C. burmannii* doses increased the SOD level. The antioxidant defense system stimulates the cellular repair response in the presence of oxidative stress [16]. SOD plays an important role in protecting cells from oxidative stress. Oxidative stress is initiated by an increase

in the production of free radicals. Auto-oxidation of glucose can decrease the concentration of low-molecular-weight antioxidants in tissue and disrupt the enzymatic activity of antioxidant defenses [17]. In DM patients, a decrease in the SOD level is related to production of various markers of oxidative stress, such as lipid hydroperoxide, conjugated dienes and protein carbonyl [18]. *C. aurantifolia* contains high levels of antioxidants. Evaluation of SOD activity in 140 types of plants showed very high SOD activity in *C. aurantifolia* and *C. burmannii* [19].

C. aurantifolia contains chemical compounds such as citric acid, essential oils (citral, limonene, felandren, *C. aurantifolia* camphor, kadinen, geranilasetat, linalilasetat, aktilaldehid and nonildehid), vitamin C and flavonoids (hesperidin, hesperetin 7-rutinoside, tangeretin, naringin, eriocitrin and eriocitrocide) [20]. Vitamin C (ascorbic acid) is an inhibitor of the enzyme aldose reductase.

Another benefit of antioxidants is that they minimize the formation of advanced glycation end products (AGEs) in the polyol pathway (sorbitol–aldose reductase pathway). A reduction in tissue accumulation of sorbitol would suppress fructose synthesis; thus, the process of non-enzymatic glycosylation would also be suppressed. Ascorbic acid works extracellularly in blood vessels and intracellularly in endothelial cells. Extracellularly, this antioxidant suppresses formation of superoxide radicals, which are formed during the glucose auto-oxidation process [21]. Some citrus species such as *C. aurantifolia, Citrus aurantium, Citrus sinensis* and *Citrus grandis* have been confirmed to have anti-diabetic properties [22]. The flavonoids in *C. aurantifolia* juice were found to reduce oxidative stress in diabetic rats [23].

Hyperglycemic conditions increase the oxidation of NAD(P)H, which induces the production of free radicals. Excessive free radical production triggers oxidative stress and damages blood vessels [6]. Under these conditions, the smooth muscle cells of blood vessel walls become damaged, leading to decreased SOD production [7]. *C. burmannii* is also capable of decreasing blood glucose levels [24]. *C. burmannii* contains methylhydroxychalcone polymer (MHCP) and other substances that increase the uptake of glucose and insulin receptor phosphorylation [23].

The analysis of LDL levels showed that the extracts of *C. aurantifolia* and *C. burmannii* had a significant effect on cholesterol levels. Lime extract contains citric acid, which can lower the pH in the digestive tract. Acidic conditions in the gastrointestinal tract stimulate the formation of bile salts to neutralize the acid. Bile salts are the end product of cholesterol metabolism. Under acidic conditions in the digestive system, more cholesterol is metabolized, and the blood cholesterol level decreases [25]. Khan *et al* [24] also found that *C. aurantifolia* lowered LDL levels in blood. In the treatment group K5, the acidic conditions in the digestive tract stimulated the pancreas to increase the production of liquid sodium bicarbonate formed from cholesterol, leading to a decrease in the blood cholesterol level. The blood cholesterol showed a direct relationship with the LDL level (the higher the blood cholesterol level, the higher the level of LDL and vice versa).

Compared with the control group (K1), higher LDL cholesterol levels were observed in rats with DM that did not receive extract treatment (K2 group). This was due to the high-fat diet given to K2 rats to induce hyperglycemia (DM), which also increased the LDL level. Furthermore, DM patients also suffer from dyslipidemia [23], because DM is associated with altered HDL cholesterol levels due to HDL catabolism. Increased HDL catabolism occurs due to an increase in hepatic lipase activity in liver cells. Glucose levels can be used as an indicator of increased hepatic lipase activity. An increase in glucose levels can occur as a result of insulin resistance [22].

C. aurantifolia contains flavonoid compounds such as naringin and hesperidin, which can increase the extent of hyperlipidemia in animals with type 2 DM by regulating fatty acid and cholesterol metabolism. *C. aurantifolia* extract also affects the expression of genes encoding enzymes involved in glucose metabolism [20].

C. burmannii contains MHCP, an active compound that acts as an insulin mimic. The mechanisms affected by MHCP include insulin receptor phosphorylation, glucose uptake and glycogen synthesis [22]. In DM patients, MHCP may decrease insulin resistance, which in turn can help control the release of non-esterified fatty acids from adipose tissue and increase the activation of lipoprotein lipase in adipose tissue [24].

CONCLUSION

The combination of *C. aurantifolia* and *C. burmannii* extracts may be a promising alternative preventative medicine for management of diabetic complications in patients with hyperglycemia.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claim relating to the content of this article will be borne by the authors. Herin Mawarti wrote the manuscript and analyzed the data. Khotimah analyzed the data and reviewed the paper. Mohammad Zulfikar As'ad designed the research and interpreted the data. Mukhammad Rajin designed the research and wrote and reviewed the paper. All authors participated in the design of the study data, read and approved the final manuscript.

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