In Vitro and *In Vivo* Antiglycation Effects of *Connarus ruber* Extract

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ABSTRACT

Accumulation of advanced glycation end products (AGEs) of the Maillard reaction has been implicated in the pathogenesis of diabetes and its complications. *Connarus ruber* has been used as a folk remedy for several diseases, including diabetes; however, its underlying mechanism has not yet been investigated. This study investigated the effects of *C. ruber* extract against glycation on collagen-linked AGEs *in vitro* and streptozotocin-induced diabetic rats (STZ-DM rats) *in vivo*. The antiglycation activities of *C. ruber* extract and aminoguanidine (AG) were examined using a collagen glycation assay kit. Nonfluorescent AGE, N^{ε}-carboxymethyl lysine (CML), N^{ω}-carboxymethyl arginine, and N^{ε}-carboxyethyl lysine levels were measured via electrospray ionization-liquid chromatography-tandem mass spectrometry. The effect of the extract on the cytotoxicity of methylglyoxal (MG), a precursor of AGEs, was examined in HL60 cells. STZ-DM rats were treated with the extract for 4 wk, and the effect was assessed using biochemical markers in the serum and CML-positive cells in renal tissues. *C. ruber* extract dose-dependently inhibited the glycation of collagen and formation of nonfluorescent AGEs, which was comparable to AG, and it significantly attenuated MG-induced cytotoxicity in HL60 cells. Furthermore, the glycated albumin levels in STZ-DM rats decreased, the increase in serum lipid levels was reversed, and immunohistochemistry demonstrated that CML deposition in the glomerulus of STZ-DM rats significantly decreased. Although further studies are needed, *C. ruber* could be a potential therapeutic for preventing and progressing many pathological conditions, including diabetes.

Introduction

The formation of AGEs begins from the nonenzymatic Maillard reaction (called glycation) between reducing sugars and free amino groups of proteins, lipids, or nucleic acids. It leads to a succession of rearrangements of intermediate compounds, the conversion of reversible Schiff's base adducted to the covalently bound Amadori products, and ultimately to the irreversibly bound products known as AGEs. Glycation-induced biological products, glycated intermediates, and AGEs are associated with physiological and

ABBREVIATIONS

AG	aminoguanidine
AGE	advanced glycation end product
ALT	alanine aminotransferase
CEL	N^{ε}-carboxyethyl lysine
CMA	N^{ω}-carboxymethyl arginine
CML	N^{ε}-carboxymethyl lysine
DM	diabetes mellitus
EZR	Easy R software
HDL	high-density lipoprotein
LDL	low-density lipoprotein
MG	methylglyoxal
ROS	reactive oxygen species
STZ	streptozotocin

pathological processes such as diabetes and aging [1]. The undesirable effects are related to their ability to promote oxidative stress and inflammation via binding to the AGE receptors on the cell surface or crosslinking with other proteins resulting in the alteration of their structure and function [2]. Thus, the potential strategies to target harmful glycation are inhibiting the Maillard reaction or breaking the existing intermediates and AGE crosslinking using specific materials [3, 4].

Collagen is the most abundant polypeptide in connective tissues such as the tendons, skin, bone, cartilage, or vessels [5]. It has a long biological half-life, and the extent of collagen glycation gradually increases in hyperglycemic conditions [6]. Among the 26 different types of collagens, the fibril-forming type I collagen, which is mainly found in the skin and tendons, is often used for both *in vitro* and *in vivo* studies of glycation.

Hundreds of AGEs and glycation intermediaries have been discovered. Only a few AGEs, specifically CML, CEL, CMA, and a few glycation intermediaries, MG, hydroimidazolones, and pentosidine, have been reported as *in vivo* collagen modifications [7, 8]. CML is an abundant and well-characterized noncrosslinking, nonfluorescent AGE, commonly known as a biomarker of oxidative stress and long-term protein damage [9]. MG is a highly reactive electrophilic α, β-dicarbonyl aldehyde compound formed mainly during glycolysis, which attacks the lysine and arginine residues of long-lived proteins, such as collagen, resulting in the formation of irreversible AGEs [10]. MG can increase ROS production, cause oxidative stress, and show significant cytotoxic effects [11].

DM is one of the world's most common chronic metabolic disorders and the most important cause of healthcare expenditure, disability, and mortality. The characteristic of DM is chronic hyperglycemia, accompanied by an accelerated rate of AGE formation that plays an important role in the pathogenesis of diabetic complications [12]. Diabetic nephropathy (DN) is one of the most common diabetic complications and the leading cause of end-stage renal failure. The level of CML increases in the serum and organs of diabetic patients [13], and its increase has been recognized as a critical step in the pathogenesis of insulin resistance, dyslipidemia, and diabetic complications [14].

STZ, an analog of N-acetylglucosamine with a molecular weight of 265.221 g/mol, inhibits DNA synthesis and is toxic to cells. It is similar to glucose and is transported into cells by glucose transporter 2 (GLUT2) but is not recognized by the other glucose transporters. β cells in the pancreas have relatively high GLUT2 levels, and it is for this reason that STZ has specific toxicity for β cells. Insulin deficiency induced by STZ treatment causes hyperglycemia and mimics the diabetic pathology. Since 1963, when the diabetogenic action of STZ was first reported [15], it has been used to create many animal diabetes models.

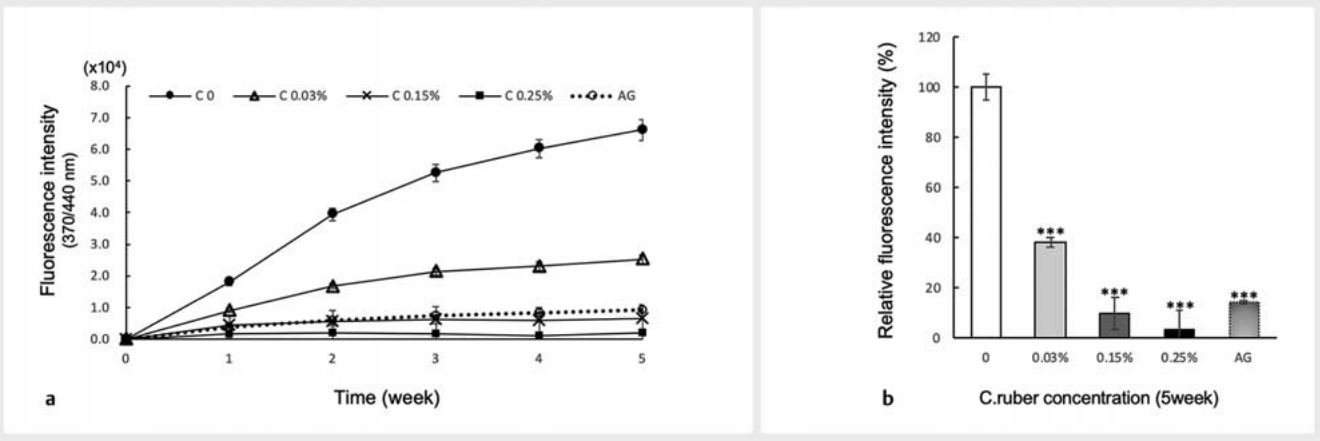
Connarus ruber (Poepp.) Planch, belonging to Connaraceae, a shrub, liana, and triphyllous species, is distributed throughout South America, especially in the middle of the Amazon. *C. ruber* has been used as a folk remedy for several diseases, including diabetes in South America, and its extract is consumed as herbal tea. Previously, we reported that its extract decreased the level of DNA damage in the white blood cells of smokers [16] and prevented the induction of insulin resistance [17]. However, the underlying mechanism has not yet been elucidated. The present study aimed to investigate the effect of *C. ruber* extract on glycation *in vitro* and *in vivo*.

Results

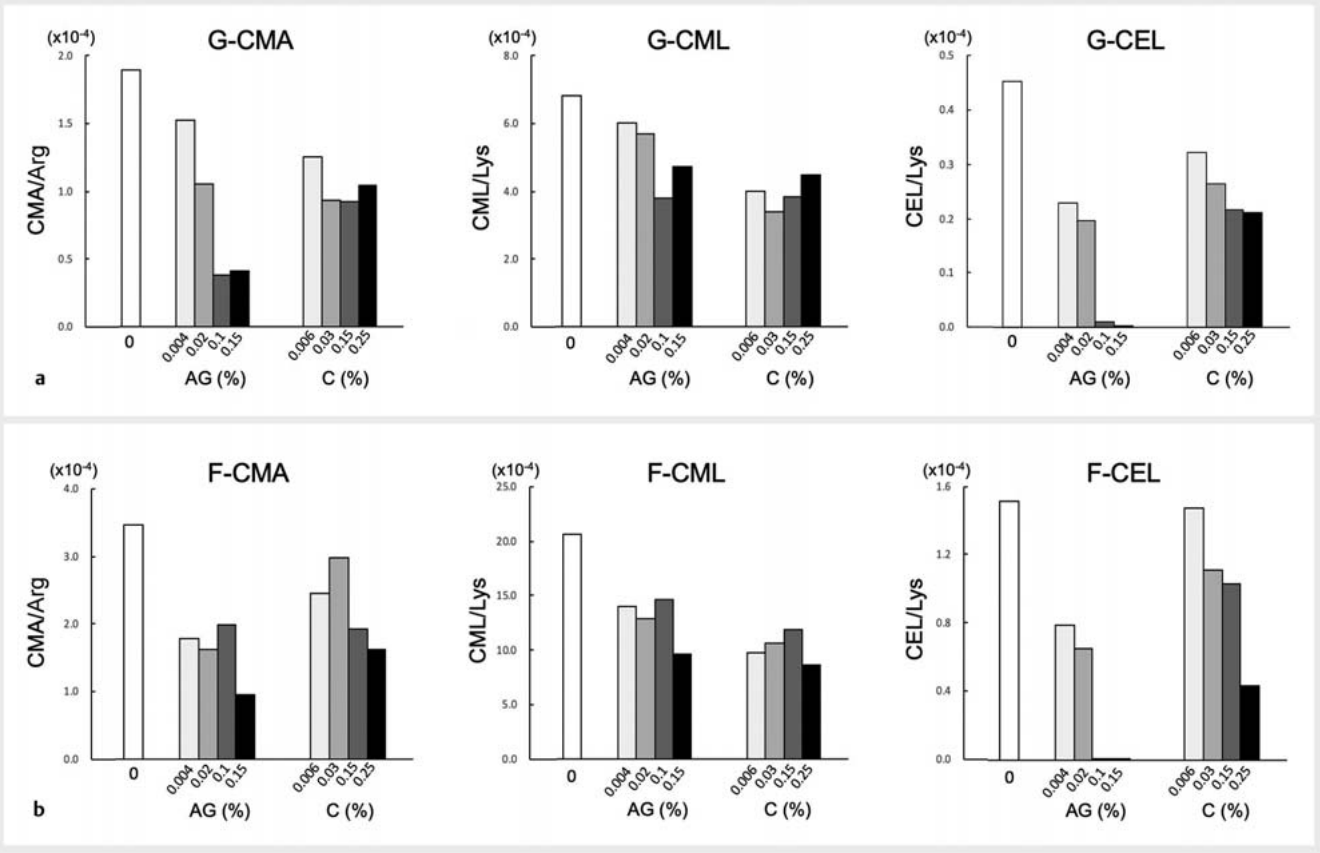
The levels of glycated collagen in various sugars were first analyzed. The highest levels were found in fructose, followed by maltose, dextran, galactose, lactose, glucose, and mannitol (data not shown). Glucose, the body's most prevalent reducing sugar, and fructose, possessing the highest glycation potential among those examined, were used for subsequent analyses. *C. ruber* extract inhibited the formation of fluorescent AGEs with 200 mM fructose in a dose-dependent manner (▶ **Fig. 1a**). Incubation with 0.03% *C. ruber* extract for 5 wk significantly decreased the fluorescence intensity of AGEs to 38.2 ± 2.03% compared to the control value ($p < 0.001$). Additionally, the fluorescence intensity of 0.15% *C. ruber* extract (9.86 ± 6.58%) was comparable to that of 20 mM (0.15%) AG, an inhibitor of glycation, often used as a positive standard in *in vitro* experiment (14.19 ± 0.66%) (▶ **Fig. 1b**). These results demonstrated that *C. ruber* extract produced dose-dependent antiglycation effects on collagen glycation via inhibition of the formation of fluorescent AGEs.

To further examine whether *C. ruber* extract could inhibit fluorescent and nonfluorescent AGEs formation, the amounts of CML, CMA, and CEL in collagen samples incubated with glucose or fructose for 23 days were measured using 3200Q LC/MS/MS. *C. ruber* extract treatment suppressed the formation of these nonfluorescent AGEs. After using 0.03% *C. ruber* extract, the glucose-induced formation of CMA decreased to 49.4%, CML to 49.5%, and CEL to 58.1% compared to the control (▶ **Fig. 2a**). Similar results were observed for fructose (▶ **Fig. 2b**). The inhibitory effects of *C. ruber* extract treatment were comparable to those of AG, and these results demonstrated that it inhibited the formation of both fluorescent and nonfluorescent AGEs. It should be noted that *C. ruber* extract is a mixture containing many components, and AG is a single component.

Subsequently, we examined whether *C. ruber* extract could influence the cytotoxicity of MG, a glycation intermediate and pre-



▶ **Fig. 1** Effect of *C. ruber* extract on glycated collagen. Collagen was incubated with 200 mM fructose and different concentrations (0–0.25%) of *C. ruber* extract or 20 mM (0.15% W/W) AG at 37 °C for 5 wk under high humidity. The glycation levels of collagen were assessed every week by measuring the intensity of advanced glycation end products (AGE)-specific fluorescence (at 370 nm excitation and 440 nm emission) (**a**). The intensity at wk 5 was expressed as a percentage of the intensity of collagen without *C. ruber* extract (**b**). Data are presented as mean ± standard deviation of 4 independent experiments. C; *C. ruber* extract, AG; aminoguanidine. *** indicates $p < 0.001$ as compared to the untreated control.



▶ **Fig. 2** Effect of *C. ruber* extract on the formation of nonfluorescent AGEs. Collagen samples were incubated with 200 mM glucose (**a**) or fructose (**b**) and different concentrations of *C. ruber* extract (0–0.25% W/W) or aminoguanidine (0–0.15% W/W) at 37 °C for 23 days under high humidity. Each sample was hydrolyzed with hydrochloric acid at 110 °C for 20 h, and the contents of advanced glycation end products (AGEs) were estimated using 3200Q LC/MS/MS. The lysine-derived AGE contents (CML, CEL) were normalized using the lysine content, and the arginine-derived AGE content (CMA) was normalized using the arginine content. G; glucose, F; fructose, C; *C. ruber* extract, AG; aminoguanidine

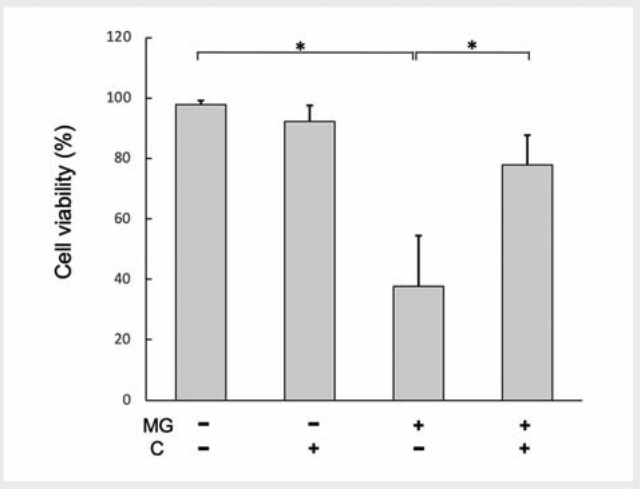
cursor of AGEs, on HL60 cells, a human promyelocytic leukemia cell line. MG significantly decreased the cell viability to 37.7 ± 16.8 % compared to the control value ($p < 0.05$). This decrease was significantly recovered to 77.69 ± 9.8 % by *C. ruber* extract ($p < 0.05$) (▶ **Fig. 3**). These results demonstrated that *C. ruber* extract treatment suppressed the cytotoxic effects of MG on HL60.

We examined the effects of *C. ruber* extract *in vivo*. Rat diabetes models were produced using STZ; *C. ruber* extract or water was orally administered to nondiabetic rats (nonDM rats) and STZ-induced diabetic rats (STZ-DM rats). STZ-DM rats showed a more significant reduction in body weight than nonDM rats after 5 wk ($p < 0.001$). *C. ruber* extract treatment had no impact on nonDM rats, but it significantly increased the body weight of STZ-DM rats ($p < 0.05$) (▶ **Fig. 4a**). Biochemical analysis was performed in the serum samples after 1 mo from the start of the treatment. We first focused on whether STZ treatment affected blood chemistry besides serum glucose levels (▶ **Fig. 4b–l**). The serum albumin levels were slightly decreased ($p < 0.05$). Serum urea ($p < 0.05$), alanine-aminotransferase (ALT) ($p < 0.005$), total cholesterol ($p < 0.005$), triglyceride ($p < 0.05$), LDL cholesterol ($p < 0.05$), HDL cholesterol ($p < 0.005$), and glycated albumin ($p < 0.005$) levels were significantly increased. These changes were similar to those previously observed [18, 19] and those seen in early-stage human diabetic patients [20], indicating the successful diabetogenic action of STZ. We next focused on whether *C. ruber* extract treatment affected the blood chemistry of nonDM rats. Serum ALT levels were significantly increased, but the values were within the baseline. No other significant differences were observed after *C. ruber* extract treatment (▶ **Fig. 4b–l**). Following this, we focused on whether *C. ruber* extract treatment had any effect on STZ-DM rats. It significantly decreased total cholesterol ($p < 0.05$), triglyceride ($p < 0.005$), and LDL cholesterol ($p < 0.05$) almost to normal levels. It also significantly lowered glycated albumin levels ($p < 0.05$). Meanwhile, it should be noted that *C. ruber* extract did not decrease HDL cholesterol or glucose levels (▶ **Fig. 4b–l**).

We then histologically examined the renal sections of experimental animals. In the glomerulus, cells with CML deposition throughout the cytoplasm were observed. These CML-positive cells were found in some glomeruli of all rats (▶ **Fig. 5a–d**), but the number of CML-positive cells was higher in STZ-DM rats and decreased with *C. ruber* extract treatment (▶ **Fig. 5a–d**). Among these cells, those with round and clear nuclei were mesangial cells, and the elongated cells with flattened nuclei were endothelial cells. The glomerulus in which CML deposited mesangial cells were found was defined as AGE-positive glomerulus, and the number of AGE-positive glomeruli was counted. Quantitative analysis showed that the ratio of AGE-positive glomeruli of STZ-DM rats was significantly higher than that of nonDM rats ($p < 0.05$), and *C. ruber* extract treatment significantly reduced the number of AGE-positive glomeruli ($p < 0.05$) (▶ **Fig. 5e**). These results showed that *C. ruber* extract inhibited the deposition of CML in the kidneys, not only in degree but also in number.

Discussion

The present study focused on *C. ruber*, a plant from the Connaraceae family that grows in the middle of the Amazon and is consumed as herbal tea. Various natural products, including tea leaves, herbs, and oriental medicines, have been reported to inhibit the Maillard reaction [21]. We first assessed the inhibitory effects of *C. ruber* on the Maillard reaction product formation by determining the AGEs generated via the interaction of glucose and fructose with collagen.

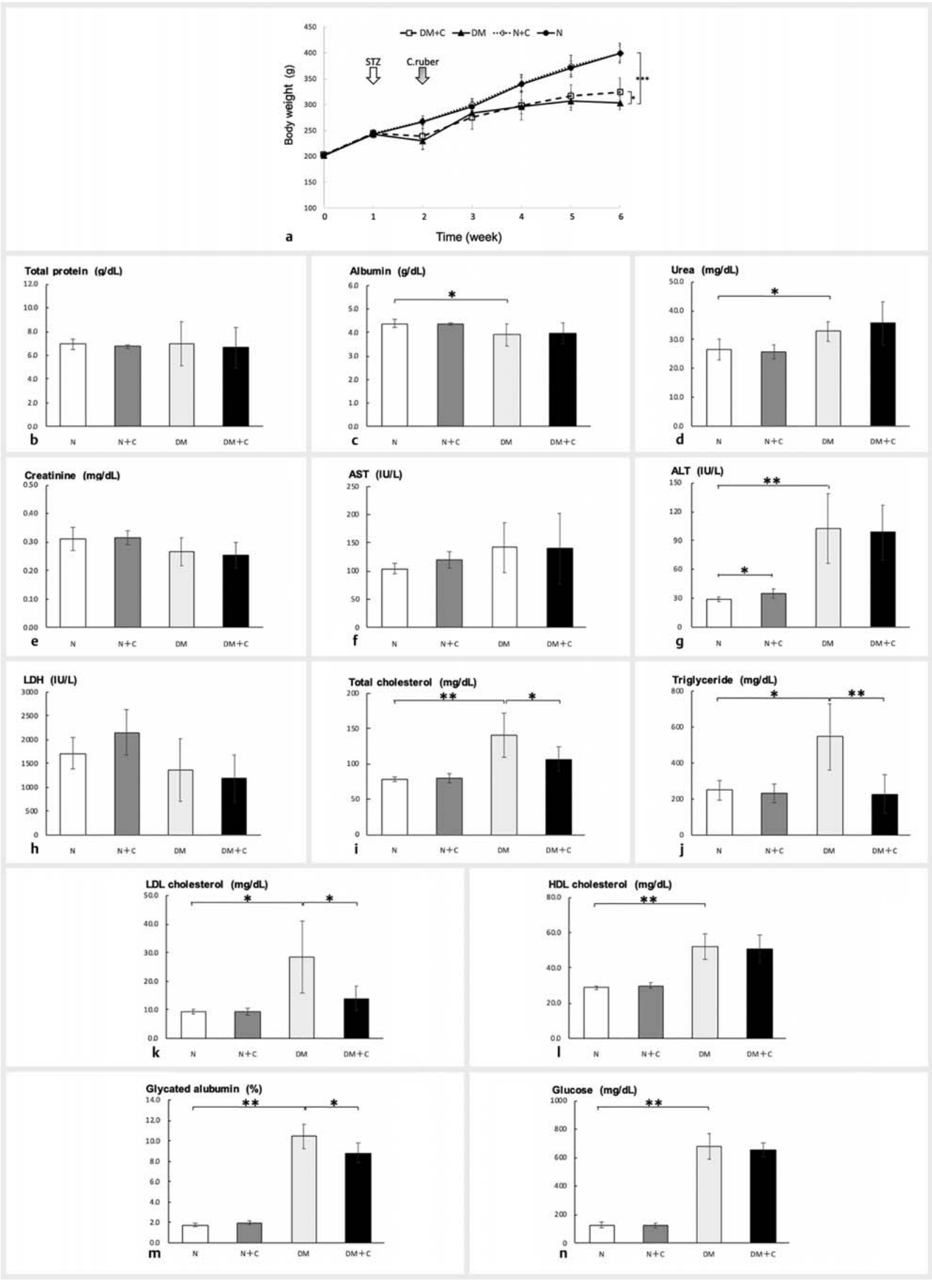


▶ **Fig. 3** Effect of *C. ruber* extract on the cytotoxicity of methylglyoxal on HL60 cells. HL60 cells (1×10^4 cells) were cultured with 50 mM methylglyoxal with or without 0.15 % *C. ruber* extract at 37 °C for 2 days. Cell viability was determined using the trypan blue dye exclusion test. Data are presented as the mean ± standard deviation of 3 independent experiments. C; *C. ruber* extract, MG; methylglyoxal. *$p < 0.05$.

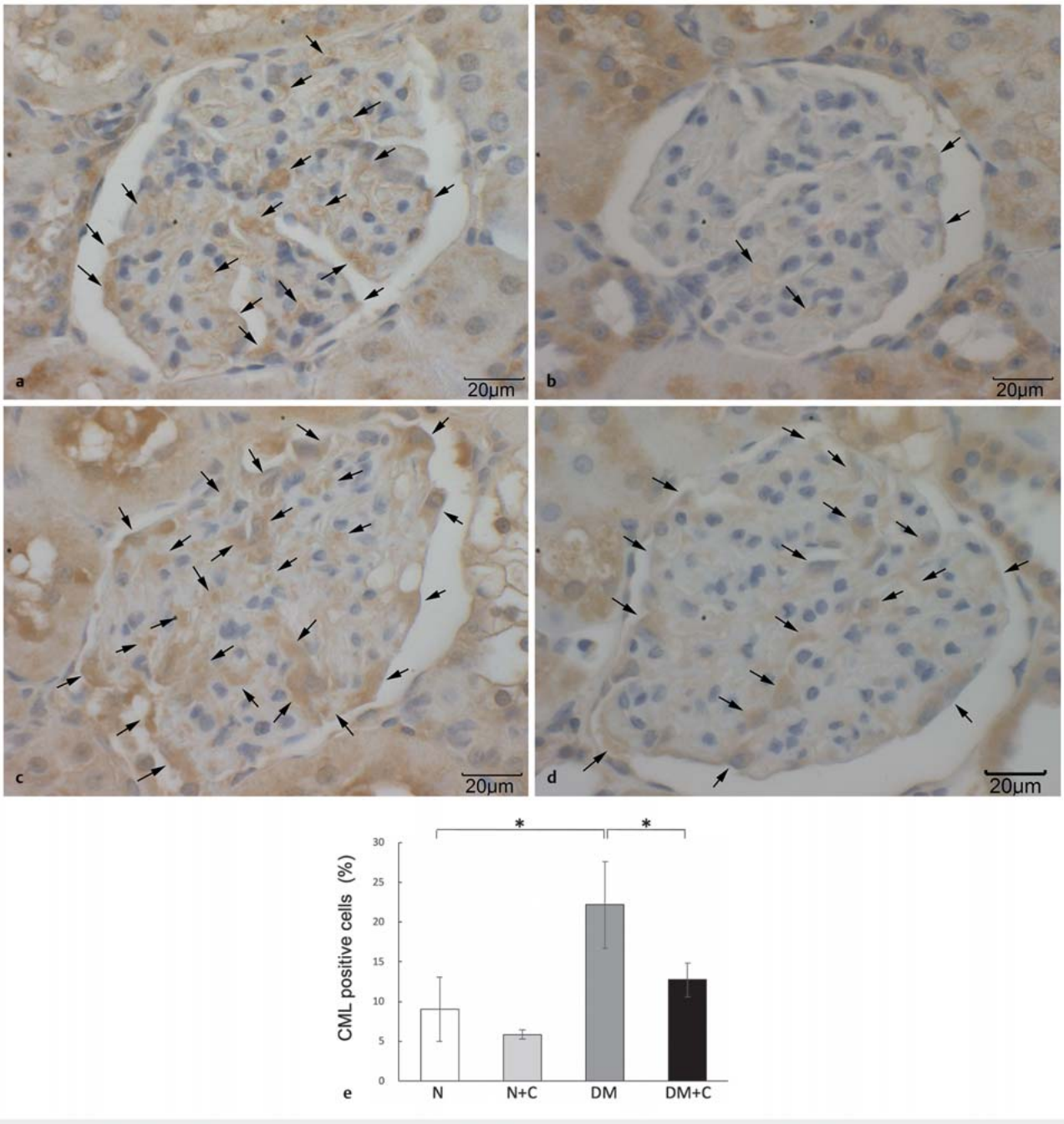
Collagen, a structural protein that helps in the formation of organs such as the skin, bone, and cartilage, is especially exposed to glycation because collagenous proteins contain several lysine, hydroxyl lysine, and arginine residues with free amino groups that have a very slow turnover rate with a half-life of 15 y. This makes them susceptible to glycation, eventually resulting in the accumulation of AGEs [22]. Therefore, the level of glycated collagen provides a measure of the formation of AGEs. In this study, we demonstrated that *C. ruber* showed antiglycation effects in a dose-dependent manner, which were assessed by the decrease in the levels of fluorescent and nonfluorescent AGEs (CML, CMA, and CEL) by glycated collagen.

MG, a reactive α-dicarbonyl compound, is one of the most powerful glycating agents of proteins and other important cellular components that form AGEs. MG shows significant cytotoxic effects by inducing apoptosis via the generation of ROS [23], induction of oxidative DNA damage [24], or accumulation of specific MG-derived AGEs [25]. Recent studies have reported that many diseases such as diabetes, cataracts, hypertension, and uremia are intimately linked to MG-derived AGE formation [26]. The present study showed that *C. ruber* could inhibit the cytotoxic effects of MG at least on HL60 cells.

STZ administration causes the reduction of functional β cell mass that manifests as insulin deficiency and subsequent inability to handle glucose, including hyperglycemia and AGEs formation.



◀ ▶ **Fig. 4** Effect of *C. ruber* extract on the body weight and blood chemistry analysis of experimental animals. The body weight of all experimental animals was measured every week (**a**). After the induction of diabetes mellitus (DM) using streptozotocin (STZ), *C. ruber* extract was administered to nonDM and STZ-induced DM rats. After 4 wk, biochemical analyses, including the measurement of glucose and glycated albumin levels, were performed in rats (**b**–**n**). Data are presented as the mean ± standard deviation; n = 5/N and N + C, n = 10/DM and DM + C. N; nonDM rats, N + C; *C. ruber* extract-treated nonDM rats, DM; STZ-induced DM rats, DM + C; *C. ruber* extract-treated STZ-induced DM rats. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.



▶ **Fig. 5** Effect of *C. ruber* extract on AGE deposition in the glomerulus of experimental animals. CML-stained sections of representative glomerulus of the 4 groups at an original magnification of 400×. Scale bars show 20 μm. Arrows indicate CML-positive lesions (**a**–**d**). The ratio of the number of CML-positive glomeruli to the total number of glomeruli were compared. The glomerulus in which CML deposited mesangial cells was found was defined as CML-positive glomerulus (**e**). Data are presented as the mean ± standard deviation; N; nonDM rats, N + C; *C. ruber* extract-treated nonDM rats, DM; STZ-induced DM rats, DM + C; *C. ruber* extract-treated STZ-induced DM rats. $^{*}P < 0.05$.

Although many animal diabetes models have been created by STZ, no standard protocol exists for the preparation [27]. The administration concentration and method of STZ differ depending on the experiment [28]. We used a single intraperitoneal injection of STZ with 75 mg/kg BW to create rat DM models because more than 80 % of rats reach hyperglycemia with more than 250 mg/dL of serum glucose; thereafter, we examined the effects of *C. ruber in vivo*. STZ-DM rats showed a significant reduction in body weight compared to nonDM rats. *C. ruber* had no impact on nonDM rats, but it significantly increased the body weight of STZ-DM rats.

Conventional diabetes drugs that promote insulin secretion or increase insulin activity may cause obesity, while diabetes drugs that suppress sugar absorption or increase sugar excretion from the kidney may lead to weight loss. However, *C. ruber* has less risk of these weight changes. Since keeping animals in metabolic cages was not allowed ethically by the animal commission, we could not perform a quantitative urinalysis. Qualitatively, polyuria, high glycosuria (+++), and proteinuria (+) were observed with STZ-DM rats (data not shown). On the other hand, a quantitative blood examination was performed. For nonDM rats, *C. ruber* induced few changes on the examined serum biomarkers. ALT levels were statistically increased, but the values were still within the baseline. These observations partially support the safety of *C. ruber's* oral administration; however, more studies should be conducted to validate this fully.

Diabetic patients show lipid abnormalities, often termed "diabetic dyslipidemia". It is typically characterized by high total cholesterol, high triglycerides, low HDL cholesterol, and increased levels of small dense LDL particles [2, 3]. Dyslipidemia combined with elevated blood glucose levels increases atherosclerosis-related inflammation, causes microvascular damages [29], and finally induces 3 major diabetic complications, namely diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy [30, 31]. Our results indicate that *C. ruber* may prevent these complications through normalizing lipid abnormalities. It remains unclear whether *C. ruber* is effective for nondiabetic dyslipidemia such as familial hypercholesterolemia.

In this study, the glycated albumin level was measured as an index of *in vivo* glycation. Albumin is highly sensitive to glycation due to its higher serum concentration than other serum proteins [32]. Glycated albumin represents the average level of blood glucose in the recent 2–3 wk. Unlike blood glucose, it is not affected by food prior to blood collection. Additionally, unlike HbA1c, which represents the average level of blood glucose in 2–3 mo, glycated albumin is neither affected by changes in the erythrocyte survival time nor is it subjected to interference by endogenous glycated amino acids [33]. Glycated albumin has also been evaluated as a valuable marker for assessing the risk of atherosclerosis and microvascular conditions and predicting the degree of diabetic complications [34, 35]. In this study, *C. ruber* significantly reduced the increase of glycated albumin levels of STZ-DM rats. Besides those already mentioned in the results section, blood glucose levels were measured regularly, and there were no significant differences between *C. ruber* treated and nontreated rats (data not shown). These results strongly indicate that the antiglycation effect of *C. ruber in vivo* is not due to the decrease of blood glucose levels but due to the direct inhibition of glycation, as in the case of *in vitro* results. Many folk remedies, as well as traditional diabetes drugs, aim to lower blood sugar levels. As already mentioned, it is achieved by increasing insulin secretion, enhancing glucose uptake by adipose and muscle tissues, inhibiting glucose absorption from the intestine and glucose production from hepatocytes, and increasing sugar excretion from the kidneys [36]. *C. ruber* is the first agent that decreased the glycated albumin levels without showing a significant reduction in glucose levels. *C. ruber* could reduce the risk of sudden death from hypoglycemia, one of the acute complications of diabetes.

Accumulation of glycated proteins is mainly associated with aging, neurodegenerative disorders, diabetes and its complications, atherosclerosis, renal failure, immunological changes, retinopathy, skin photoaging, osteoporosis, and progression of some tumors [1]. Yang et al. (2017) suggested that CML might cause diabetic nephropathy by disturbing the intracellular feedback regulation of cholesterol metabolism, resulting in hyperlipidemia and lipid accumulation. Therefore, inhibition of CML-induced lipid accumulation might play a potential renoprotective role in the progression of diabetic nephropathy [37]. *C. ruber* ameliorated the dyslipidemia observed in STZ-DM rats. CML is filtered through the glomeruli, and it is reabsorbed in the proximal tubules [38]. The decreased glomerular filtration rate and tubule cell damage could also be involved in CML accumulation [39]. *C. ruber* clearly reduced the deposition of CML in the glomerulus, and the decrease was statistically significant in STZ-DM rats. However, it is unclear whether the systemic production of CML was suppressed by *C. ruber*, which led to reduced CML deposition, or CML deposition was reduced due to increased glomerular filtration rate by *C. ruber*.

In the present study, we reported preliminary data on antiglycation effects of *C. ruber in vitro* and *in vivo*. Although further studies should be conducted on which components contained in *C. ruber* show antiglycation effects and the mechanisms through which they act, *C. ruber* will be a useful seed for further antiglycation research and a potential therapeutic for the prevention and progression of many pathological conditions, including DM.

Materials and Methods

Materials

C. ruber harvested in the Amazon with the permission of Instituto Nacional de Pesquisas da Amazônia (INPA) was purchased from Agrorisa. The Collagen Glycation Assay Kit was purchased from Cosmo Bio. MG was purchased from NACALAI TESQUE, and STZ was purchased from Sigma-Aldrich. CMA and CML were purchased from Nippi. ($^{13}C_6$,$^{15}N_4$)-Arg and ($^{13}C_6$,$^{15}N_4$)-Lys were purchased from Cambridge Isotope Laboratories. CEL and (d_4)-CEL were purchased from PolyPeptide. ($^{13}C_6$,$^{15}N_4$)-CMA and ($^{13}C_6$,$^{15}N_2$)-CML were synthesized as previously described [40, 41]. AntiCML mouse monoclonal antibody (CMS-10) was purchased from TransGenic, horseradish peroxidase (HRP)-labeled goat antimouse IgG polyclonal antibody from Nichirei Bioscience, and 3,3′-diaminobenzidine from DAKO. All other chemicals were of the best grade available from commercial sources. HL60 was obtained from RIKEN BioResource Research Center.

Preparation of *C. ruber* extract

The inner bark taken from *C. ruber* was dried and crushed, 4 g of which was soaked in 80 mL of hot water (80 °C) for 30 min with gentle stirring, followed by filtration. In order to measure the solid content of the filtrate, half of the filtrate was concentrated under reduced pressure, and 0.1 g of solid content was obtained. Based on the result, the aqueous solution obtained from 4 g of the inner bark and 80 mL of hot water was treated as 0.25 % *C. ruber* extract in the subsequent experiment.

Glycation assay

The effects of *C. ruber* extract on collagen glycation were assessed by measuring fluorescent AGEs using the Collagen Glycation Assay Kit, which contained skin-derived type I collagen. Collagen solution (50 µL, stored on ice < 10 °C before testing) was added to a 96-well black plate, and the plate was incubated at 37 °C overnight under high humidity to allow the collagen solution to gelate. Following this, 10 µL of 200 mM fructose and 40 µL of different concentrations (0–0.25 %) of *C. ruber* extract or 20 mM AG were added to each well containing collagen gel. The plate was incubated at 37 °C for 5 wk under high humidity to induce glycation. The degree of glycation was assessed by measuring the intensity of AGE-specific fluorescence (at 370 nm excitation and 440 nm emission) using a fluorescence plate reader (Perkin Elmer).

Measurement of AGEs using 3200Qtrap LC/MS/MS

Collagen solution (stored on ice at < 10 °C before testing) was added into a 1.5 mL tube, and the tube was incubated at 37 °C overnight under high humidity to allow the collagen solution to gelate. Subsequently, 250 µL of 200 mM glucose or 200 mM fructose solution and 50 µL of different concentrations (0–0.25 %) of *C. ruber* extract or 0–0.15 % of AG were added to each tube containing collagen gel, and the tubes were incubated at 37 °C for 23 days under high humidity to induce glycation. The gel was washed twice with phosphate-buffered saline (PBS, pH 7.4), hydrolyzed at 110 °C for 20 h, and concentrated via freeze-drying. The pellet was dissolved in 100 µL of water. Following this, 1 µL of 50 µM stable isotopes ((^{13}C6,^{15}N4)-CMA, (^{13}C6,^{15}N2)-CML, (d_4)-CEL) were added to each sample, 5 µL of each sample was applied to 3200Qtrap LC/MS/MS (Sciex), and the 3 types of AGEs were quantified [40].

Cell viability assay

HL60 cells, the human promyelocytic leukemia cells, were grown in RPMI 1640 medium supplemented with 10 % FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco) at 37 °C with 5 % CO_2, and the cells were used for experiments after 3 h of growth in FBS-free RPMI 1640 medium. HL60 (1×10^4 cells) were then cultured in 50 mM MG with or without 0.15 % *C. ruber* extract at 37 °C for 48 h. After incubation, the total number of viable cells was determined using the trypan blue dye (Sigma-Aldrich) exclusion test.

Animals

Six-wk-old male Sprague-Dawley rats were purchased from Japan SLC, and they were housed at 22–25 °C under 60–70 % humidity and a 12 h light/dark cycle for 2 wk for acclimation. Animals were fed standard commercial rat chow and water *ad libitum*. All animal experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the animal commission of Pharmacrea Kobe Co. Ltd (Approval number: H301214-1).

Induction of diabetes and pharmacological treatment

Diabetes was induced by a single intraperitoneal injection of STZ (75 mg/kg BW, 5 mL/kg BW) dissolved in PBS (−). Control rats were injected with PBS (−) alone. After 8 days of injection, the development of diabetes was confirmed from tail vein blood glucose level (> 250 mg/dL). The experimental animals were divided into 4 groups, namely control rats (G1), *C. ruber* extract-treated control rats (G2), STZ-DM rats (G3), and *C. ruber* extract-treated STZ-DM rats (G4) (n = 5 in G1 and G2 groups, n = 10 in G3 and G4).

C. ruber extract water was prepared by adding 40 g of the inner bark taken from *C. ruber* to 800 mL of water, and this was followed by soaking at 80 °C for 30 min under slow stirring and filtration. The treatment was performed as follows: water was administered *ad libitum* to G1 and G3, and *C. ruber* extract was administered *ad libitum* to G2 and G4. Body weight and blood glucose levels were measured at regular intervals. Diabetic and nondiabetic animals were euthanized after 4 wk from the start of treatment, and whole blood and organ sections were prepared for biochemistry and immunohistochemistry, respectively. Measurements of serum glucose, glycated albumin, total protein, albumin, creatinine, blood urea nitrogen, aspartate aminotransferase, ALT, lactate dehydrogenase, total cholesterol, triglycerides, HDL cholesterol, and LDL cholesterol levels were performed at BioMedical Laboratories.

Immunohistochemistry

Immunohistochemistry was performed on 4 µm-thick paraffin sections prepared from 10 % neutral buffered formalin (pH 7.6)-fixed rat kidneys. After dewaxing and washing, the sections were incubated with antiCML mouse monoclonal (Clone: CMS-10) antibody (KH011; TransGenic Inc) diluted to 7 µg/mL at 4 °C overnight. For visualization of reactions, HRP-conjugated antimouse IgG goat polyclonal antibody (HISTOFINE #424 134; Nichirei) and 3,3′-diaminobenzidine (#K3468; DAKO) were used.

Statistical analysis

All data are shown as mean ± standard deviation. Statistical analyses were performed using EZR [42]. A value of $p < 0.05$ was considered statistically significant.

Contributors' Statement

Mariko Takenokuchi: conceptualization, writing–original draft, preparation. Kinuyo Matsumoto: methodology, validation. Yuko Nitta: investigation, data curation. Rumi Takasugi: investigation, data curation. Yukari Inoue: investigation, data curation. Michi Iwai: investigation, methodology. Keiichi Kadoyama: investigation, software. Kazutoshi Yoshida: methodology, validation. Hiromi Takano-Ohmuro: methodology, validation. Taizo Taniguchi: conceptualization, project administration, resources.

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Conflict of Interest

Authors Rumi Takasugi, Michi Iwai, and Taizo Taniguchi of this study are regular employees of the companies. However, the funders did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. This does not alter our adherence to Planta Medica policies on sharing data and materials.

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