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Research Paper

Insulinotropic and antidiabetic properties of *Eucalyptus citriodora* leaves and isolation of bioactive phytomolecules

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Abstract

Objective The aim of this study was to delineate the mechanisms of action of the plant *Eucalyptus citriodora* used traditionally for the treatment of type 2 diabetes.

Methods Insulin secretion and signal transduction were measured using clonal pancreatic β-cells and mouse islets. Glucose uptake was assessed using 3T3-L1 adipocytes and *in vitro* systems assessed additional glucose-lowering actions. High-fat-fed (HFF) obese rats were used for *in vivo* evaluation and phytoconstituents were identified by RP-HPLC followed by LC-MS.

Key findings *Eucalyptus citriodora* stimulated 1.2–4.6-fold insulin release that was inhibited by the Ca^{2+}-channel blocker, verapamil, K_{ATP}-channel opener, diazoxide and Ca^{2+} free conditions. The effect was potentiated by IBMX and preserved in presence of tolbutamide or 30 mM KCl. The action mechanism involved membrane depolarization and elevation of intracellular Ca^{2+}. *Eucalyptus citriodora* also significantly increased glucose uptake by 3T3-L1 cells and inhibited digestion of starch, glucose absorption, DPP-IV enzyme and glycation of protein. Administration of *E. citriodora* (250 mg/5 ml/kg) for 9 days to HFF obese-diabetic rats improved glycaemic control and β-cell function. The isolated phytoconstituents responsible for the β-cell actions included quercitrin, isoquercitrin and rhodomyrtosone E.

Conclusions *Eucalyptus citriodora* improves glycaemic control via multiple mechanisms. Further studies are required to assess the utility of the plant or active constituents in the therapy of type 2 diabetes.

Keywords: insulin; glucose, type 2 diabetes; DPP-IV; phytomolecules

Introduction

Diabetes mellitus incidence is increasing globally due to obesity and increasingly unhealthy lifestyle.[1] The WHO identified the diabetes as the fastest growing metabolic disorder that has two major class namely type 1 and type 2 diabetes. Type 1 diabetes is a result of β-cell destruction, whereas type 2 diabetes is due to β-cell dysfunction and insulin resistance.[2] A report from International Diabetes Federation says that one in every 11 adults worldwide has type 2 diabetes.[3] It is expected that approximately 90% of diabetes patients have type 2 diabetes, which causes secondary complications over time such as cardiovascular disease, retinopathy, cognitive decline and end-stage renal disease.[4] The most common treatment options for type 2 diabetes are diet, weight loss and use of either single or multiple oral anti-hyperglycaemic agents. These currently include metformin, sulphonylureas, meglitinides, thiazolidenediones, DPP-IV inhibitors, GLP-1 mimetics and SGLT-2 inhibitors which enhance insulin

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action, stimulate insulin secretion or promote glucose excretion.[5] In poorly controlled cases insulin is also prescribed, but none of these interventions cure diabetes and most of these are expensive and often associated with secondary toxicities.[6] A meta-analysis showed that since 2012 prevalence of diabetes has increased by 11%, while cost per patients has increased by 13%, excluding the treatment of diabetic complications.[7] Medicinal plants and their phytochemicals have been known for many years to possess important pharmacological and biological actions.[8] In this respect, a large number of readily available and inexpensive plants have been used traditionally for treatment of obesity and diabetes and which continues today in many poorer and more remote societies.[9, 10]

Eucalyptus citriodora is a member of Myrtaceae family, and commonly known as lemon-scented gum, blue spotted gum or lemon eucalyptus. *Eucalyptus citriodora* has many traditional and pharmacological applications such as an analgesic, anti-inflammatory, and antipyretic remedy, and as an aid against respiratory infections and sinus congestion.[11] It is well known for containing important essential oils such as cineole, citronellal and citronellic acid.[12] The production of cytokines and chemokines such as TNF-a, IL-1β, leukotriene B4 and thromboxane B2 in inflammatory cells has been shown to be inhibited by eucalyptol.[13, 14] The essential oils also have potential antioxidant and inhibitory effects against a wide range of microorganisms such as bacteria, fungi and yeasts.[15, 16]

In addition to its use as traditional medicine for diabetes, *E. citriodora* has been shown to counter hyperglycaemia in alloxan-induced diabetic rats.[10] The leaves of this plant contain betulinic acid and corosolic acid which have been suggested to stimulate GLUT-4 translocation,[17] but the precise mechanisms responsible for the glucose-lowering actions are unknown. We hypothesized that phytochemicals present in *E. citriodora* exert a spectrum of anti-diabetic activities mediated through diverse mechanisms. This study was therefore sought to assess, for the first time, the various molecular pathways and phytochemicals underlying the antidiabetic actions of *E. citriodora* using a number of *in vitro* and *in vivo* models.

Materials and Methods

Collecting and preparing crude extract

Eucalyptus citriodora leaves were purchased as powdered form (botanical accession number 43755) from Jahangirnagar University, Dhaka, Bangladesh and extraction procedure was followed as per previous description.[18] Twenty-five grams of powdered leaf was dissolved into 1 l water and heated to boil. It was filtered using Whatman no. 1 filter paper and the sticky residue was then vacuum dried (Savant Speed vac, New York, NY, USA). Finally, gummy greenish residue obtained, was preserved at 4°C before analysis.

In vitro insulin-releasing studies

The clonal BRIN-BD11 cells[19] and isolated mouse islets[20] were used to test insulin-releasing effect. Leaf extract or purified HPLC fractions were incubated with or without known modulators of insulin secretion at different glucose concentrations (1.1, 5.6 or 16.7 mM). Non-stimulatory, basal and stimulatory glucose levels were described as 1.1, 5.6 and 16.7 mM, respectively. The supernatant samples were preserved at −20°C for insulin radioimmunoassay.[21] Insulin content from islets was measured by acid-ethanol extraction method.[22]

Membrane potential and intracellular calcium ($[Ca^{2+}]_i$) concentration

Both membrane potential and intracellular [Ca^{2+}]i concentrations of BRIN-BD11 cells in the presence of *E. citriodora* were measured by a FLIPR Membrane Potential and Calcium Assay Kit (Molecular Devices, Sunnyvale, CA, USA).[23]

Glucose uptake

The 3T3L1 differentiated cells were used as stated earlier.[24] Differentiated cells were treated with plant extract or 100 nM insulin at 37°C for 30 min. Then, 2-NBDG (50 nM) was extended for 5 min. With ice-cold PBS, the wells were rinsed and each slide was fixed with three to four cover slips. Images were taken using microscopy at 10× magnification, and the intensity of fluorescence was calculated.

Glycation of insulin

The *in vitro* protein glycation was followed according to previous description.[25] Human insulin (1 mg/ml) and sodium cyanoborohydride (85.3 mg/ml) were incubated with D-glucose solution (246.5 mM) with (*E. citriodora*) or without (control) treatment. The mixtures were incubated for 24 h at 37°C; finally, the reaction was stopped by 0.5M acetic acid. RP-HPLC was used to measure glycated and non-glycated insulin.[26] Positive control, aminoguanidine, a known inhibitor of protein glycation, was used.

DPP-IV enzyme activity *in vitro*

A fluorometric method was implied to determine DPP-IV enzyme activity as described previously.[27] An assay was performed using DPP-IV enzyme (8 mU/ml) and 200 µM of substrate (Gly-Pro-AMC) in a 96-well plates (Greiner). The fluorescence intensity was calculated with excitation and emission at 370 nm and 440 nm at Flex Station 3 (Molecular Devices, CA, USA) with 2.5 nm slit width. Sitagliptin, an existing DPP-IV inhibitor, was used as a positive control.

Starch digestion

In vitro assay of starch digestion was followed as stated earlier.[28] Starch solution (2 mg/ml; 100 mg in 50 ml water) was incubated without (control) or with *E. citriodora* or acarbose and 0.01% heat stable α-amylase (Sigma-Aldrich, St. Louis, MO, USA) from *Bacillus leicheniformis* was included. The resulting mixture was incubated at 80°C for 20 min. Amyloglucosidase (0.1%, Sigma-Aldrich) from Rhizopus mold was added to the diluted mixture and kept at 60°C for 30 min for further incubation. At 4°C, samples were preserved for the subsequent study of glucose release using the GOD/PAP method (Randox GL 2623).

Diffusion of glucose

Glucose absorption was assessed via cellulose ester dialysis tubing as previously reported.[29] The dialysis tube was loaded with 220 mM glucose of 2 ml volume, with or without leaf extract and the two ends of each tube were securely sealed and put in 0.9% NaCl solution of 45 ml. The glucose outside the tube was analysed after 24 h of shaking at 37°C.

Animals

A high-fat diet (Special Diet Service, Essex, UK) were fed to Sprague-Dawley male rats (Envigo UK, nearly 345–405 g) for 6 weeks before

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test. On normal diet (Trouw Nutrition, Cheshire, UK), healthy age-matched rats were preserved to use as controls. Sixteen rats were selected as diabetic model after assessing blood glucose level; rats were divided into groups including the eight age-matched lean control group (n = 8), the high-fat-fed diabetic group (n = 8) and the high-fat-fed treatment group (n = 8). According to previous studies, a single dose of extract (250 mg/5 kg, given at a volume of 5 ml/kg body weight) was chosen and was considered to reflect a high but acceptable level of ingestion that was not correlated with any adverse effects. Chronic effects of daily *E. citriodora* therapy in high-fat rats were tested at intervals over a period of 9 days without any symptoms of pain, influenced behaviour or any other adverse effects. In compliance with the UK Animals (Scientific Procedures) Act 1986 and the EU Directive 2010/63EU, all animal studies were carried out. To reduce any possible animal suffering, all appropriate precautions were taken. The animal experiments were authorized by the local Ulster Animal Welfare and Ethical Review Body (AWERB) committee (01/10/2016) as well as being protected by the PIL450, PIL1822 and PPL 2804 UK Home Office Animal project/personal license numbers, approved on 06/05/2016.

Acute oral glucose tolerance and activity of plasma DPP-IV

Glucose tolerance test (18 mmol/kg) were performed to assess glycaemic control on 6 and 12 h starved obese rats plus (250 mg/5 ml/kg *E. citriodora* leaves) or minus (control) treatment as reported earlier.[30] Blood sample was collected at indicated time points (Figure 3B and C; Figure 5A and B)) for the determination of glucose and plasma insulin levels using radioimmunoassay.[21] DPP-IV was measured by fluorometric assay.[27]

Glucose homeostasis after 9-day therapy of *E. citriodora* leaves in high-fat feeding rats

Twice-daily oral gavage with either 0.9% saline or 250 mg/5 ml/kg body weight *E. citriodora* was given to obese rats for 9 days in a raw. Intake of food and fluid, body weight, blood glucose and plasma insulin was assessed at regular intervals. After 6 days of therapy, glucose tolerance (18 mmol/kg) was tested. After chronic studies, pancreatic tissues were extracted in order to assess islet morphology and pancreatic insulin content.[30]

Determination of islet morphology

Tissue processing, fixation, staining and microscopic detection were followed as stated earlier.[31] Tissues were dissected into thin sections (5–8 μm) and fixed with 4% PFA followed by overnight incubation at 4°C in the presence of mouse anti-insulin (1:500) and guinea pig anti-glucagon (1:400) antibodies. The sections were incubated in the presence of Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-guinea pig secondary antibodies at body temperature. The slides were placed and analysed on a fluorescent Olympus System Microscope BX51 (Olympus instruments, UK) with TRITC (594 nm) or FITC (488 nm) filters following the staining of the nucleus by 4′,6-diamidino-2-phenylindole. Captured (DP70camera adapter system) images were processed using Cell^F imaging software to measure the islet, α-cell and β-cell area.

Purification of crude extracts

Leaf extract was re-suspended in 0.12% (v/v) Trifluoroacetic acid/water and various contents were separated by reversed-phase high-performance liquid chromatography using a Vydac 218TP1022 (22 × 250 mm) preparative stainless steel 10 μm C-18 column (Grace, Deerfield, IL, USA), equilibrated with 0.12% (v/v) Trifluoroacetic acid/water at a flow rate of 5 ml/min. Using linear gradients, the concentration of acetonitrile within the eluting solvent was increased to 20% for 10 min and 70% for 40 min cycle. Five fractions that showed prominent insulinotropic activity on BRIN-BD11 cells, were screened as previously stated.[32]

Structural characterization of purified extracts

LC-MS via ESI-MS technique was employed to determine molecular weights of purified peaks fractions obtained on RP-HPLC. Samples were separated using a Kinetex 5μm F5 LC column (150 × 4.6 mm, Phenomenex) on a Spectra System LC (Thermo Separation Products) and peaks were detected with a UV detection system at 220–360 nm as mentioned earlier.[33]

Statistical analysis

All statistical analyses were analysed by Graph Pad prism 5 and data interpreted as mean ± SEM with significant limit set to $P < 0.05$. An unpaired Student's t-test (non-parametric, with two-tailed P values) and one-way analysis of variance with Bonferroni post hoc tests were used to analyse all data where appropriate. The area under curve with baseline correction was plotted using the trapezoidal rules.

Results

HWEC and insulin secretion

Basal insulin secretion from clonal pancreatic β cell line, BRIN BD11 at 5.6 and 16.7 mM glucose was 1.06 ± 0.08 and 1.52 ± 0.08 ng/10^6 cells/20 min, which increased to 5.20 ± 0.2 and 7.89 ± 0.39 ng/10^6 cells/20 min ($P < 0.001$; Figure 1A and B) with the insulin secretogogues alanine (10 mM) and KCL (30 mM). Hot water extract of *E. citriodora* (HWEC) leaves significantly (P < 0.05–0.001) increased insulin release in a dose-dependent manner (1.6–5000 μg/ml) at 5.6 mM and 16.7 mM glucose from 1.54 ± 0.15 to 4.89 ± 0.14 ng/10^6 cells/20 min and 1.89 ± 0.16 to 5.87 ± 0.17 ng/10^6 cells/20 min, respectively (Figure 1A and B). At 5000 μg/ml, the extract produced a maximum increase of 3.9–4.6-fold from the basal rate ($P < 0.001$). Positive controls included insulin secretagogues, alanine (10 mM) and membrane depolarizing agent, KCl (30 mM). Importantly, extract at 1.6–200 μg/ml did not induce the release of the cytosolic enzyme, lactate dehydrogenase, suggesting plasma membrane integrity (data not shown). Hot water extract produced a substantial increase in insulin secretion from isolated mouse islets at 16.7 mM glucose (Figure 1C). Stimulation of insulin secretion by hot water extract was significant at concentrations of ≥50 μg/ml (P < 0.01–0.001). An insulin secretion modulator based on glucose, GLP-1 (10^{-6} and 10^{-8} M) and alanine (10 mM) was used as positive controls have substantially increased the release of insulin ($P < 0.001$; Figure 1C).

Eucalyptus citriodora (200 μg/ml) was incubated with BRIN-BD11 cells and known stimulators or inhibitors to evaluate the insulin releasing pathways (Figure 1E). The insulin releasing activity of extract was reduced (by 20%) in the presence of the K^+ channel opener, diazoxide (300 μM) and the L-type voltage-dependent Ca^{2+} channel blocker, verapamil (50 μM) by decreasing the intracellular Ca^{2+} influx activity (Figure 1E). Insulin-releasing activity was retained in the presence of a depolarizing 30 mM concentration of KCl. Insulin release induced by the extract was also enhanced by IBMX ($P < 0.001$; Figure 1E) and tolbutamide ($P < 0.001$; Figure

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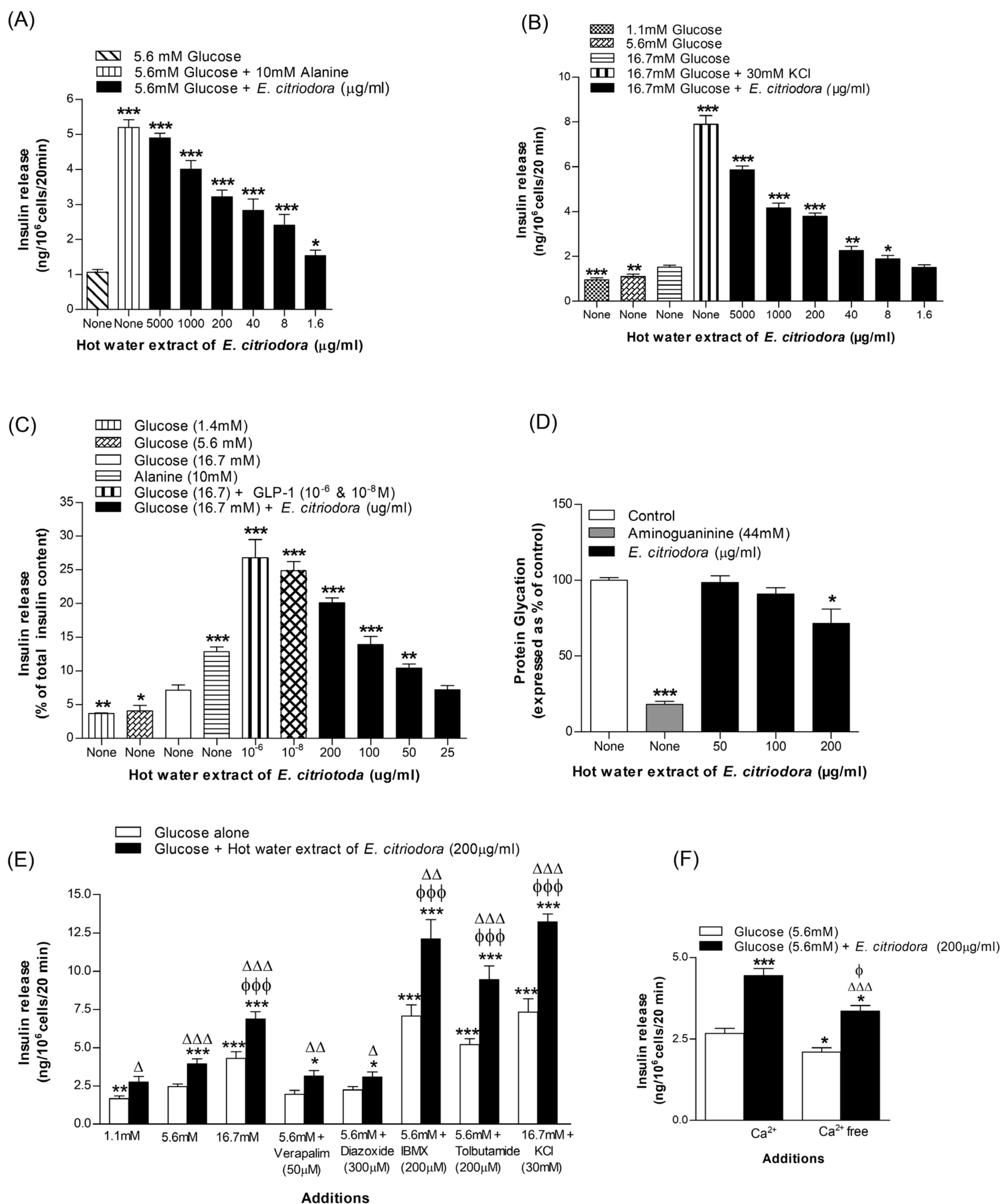

Figure 1 Concentration-dependent effects of HWEC leaves on release of insulin from (A and B) clonal pancreatic β-cell line, BRIN-BD11 cells and (C) islets of Langerhans, (D) glycation of protein, (E) secretion of insulin with known stimulators or inhibitors and (F) plus or minus extracellular calcium from clonal β-cells. Values for $n = 8$ and 4 for insulin secretion and $n = 3$ for glycation of protein are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to controls. $^{\phi}P < 0.05$ and $^{\phi\phi\phi}P < 0.001$ compared to 5.6 mM glucose with extract. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ and $^{\Delta\Delta\Delta}P < 0.001$ compared to respective incubation without extract. HWEC, Hot water extract of *E. citriodora*.

1E). The phosphodiesterase inhibitor, IBMX and sulphonylurea, tolbutamide were used to potentiate the production of cyclic AMP and secretion of insulin. In the absence of extracellular calcium, insulin release evoked by the extract was inhibited by 25% (Figure 1F).

HWEC and membrane depolarization and intracellular calcium

Plasma membrane of BRIN-BD11 cells was significantly depolarized with 30 mM KCl ($P < 0.001$). Hot water extract of *E. citriodora* (HWEC) also evoked membrane depolarization ($P < 0.001$; Figure 2A). Alanine induced a significant ($P < 0.001$) increase of intracellular calcium as did *E. citriodora* extract ($P < 0.001$; Figure 2B).

HWEC and glucose uptake and insulin action

Glucose uptake by 3T3L1 differentiated adipocytes was studied using a fluorescent glucose analogue, 2-NBDG (Figure 2C–G). *Eucalyptus citriodora* extract substantially induced glucose

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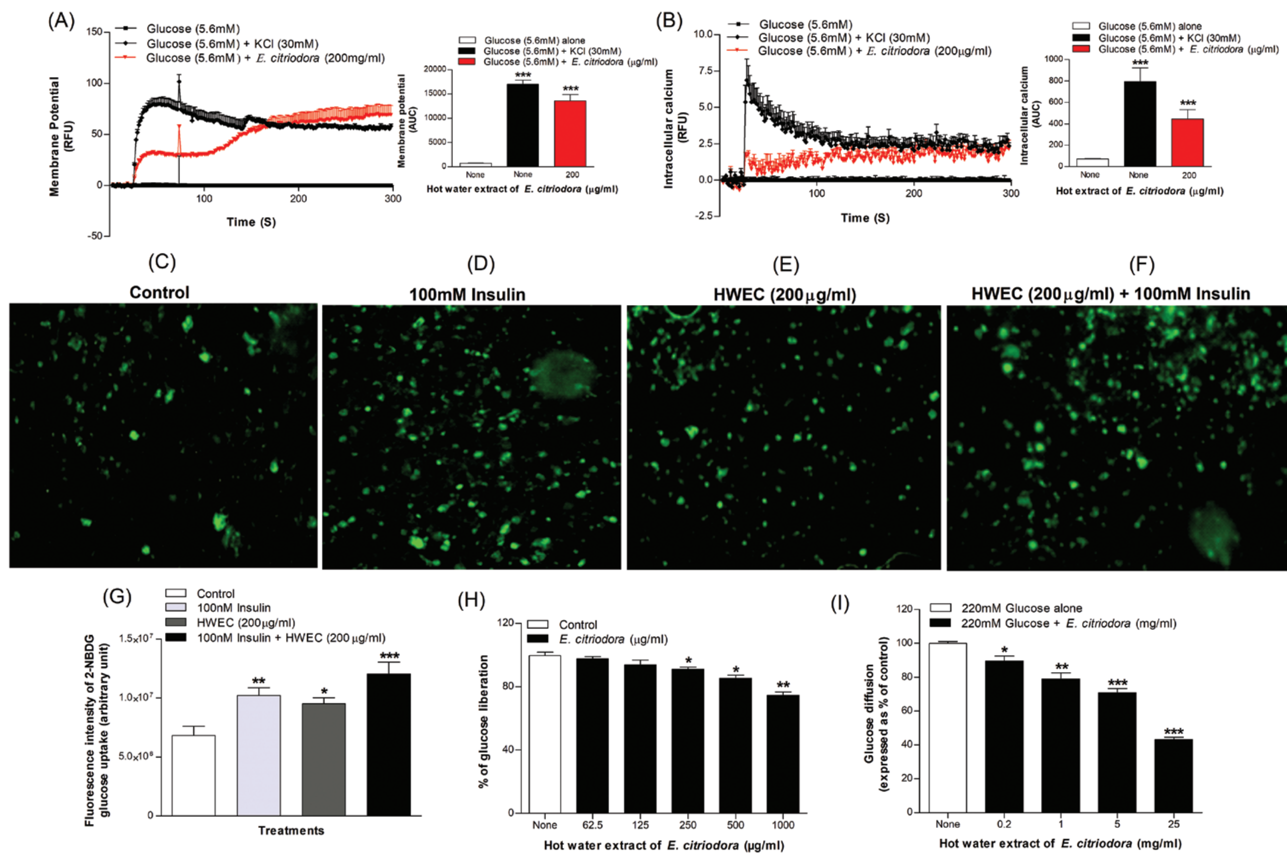

Figure 2 Effects of HWEC on (A) membrane potential and (B) intracellular calcium in clonal pancreatic β cell line, BRIN BD11 and, (C, D, E, F and G) glucose uptake, (H) starch digestion and (I) glucose diffusion *in vitro*. Changes of fluorescence intensity in differentiated 3T3L1 adipocyte incubated with HWEC (E) minus or (F) plus 100 nM insulin. The ×10 magnification was used to take the images. Values for $n = 6$ for membrane potential and intracellular calcium, $n = 4$ for uptake of glucose, digestion of starch and diffusion of glucose are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to controls.

uptake compared with control ($P < 0.05$; Figure 2E and G). This stimulatory effect was enhanced by 100 nM insulin ($P < 0.001$; Figure 2F and G).

HWEC and glycation of insulin

Hot water extract of *E. citriodora* leaves (HWEC, 200 µg/ml) significantly inhibited the glycation of insulin by 29% ($P < 0.05$–0.001; Figure 1D) in comparison to control (220 mM glucose + insulin [1 mg/ml]). Aminoguanidine (44 mM; positive control) reduced glycation of protein by 81% ($P < 0.001$; Figure 1D).

HWEC and starch digestion

The established inhibitor, acarbose (1 mg/ml) significantly decreased enzymatic glucose liberation from starch by 85% (data not shown). Hot water extract substantially ($P < 0.05$–0.01) inhibited starch digestion at concentrations >125 µg/ml, with a maximum 25% inhibition at 1000 µg/ml (Figure 2H).

HWEC and glucose diffusion *in vitro*

Eucalyptus citriodora (mg/ml) significantly inhibited glucose diffusion after 24 h of incubation (Figure 2I). A 57% inhibition was observed at the highest concentration (25 mg/ml) and the lowest effect of 10.5% was recorded at 0.2 mg/ml ($P < 0.05$–0.001; Figure 2I).

HWEC and DPP-IV enzyme *in vitro*

Sitagliptin, an established drug (10 µM), inhibited by 98% the enzymatic liberation of AMC from DPP-IV substrate, Gly-Pro-7-Amino-4-Methyl-Coumarin (data not shown). Extract remarkably inhibited DPP-IV ($P < 0.05$–0.001, Figure 3A) with a maximum effect of 75% ($P < 0.001$, Figure 3A) at 5000 µg/ml.

HWEC and oral glucose tolerance and DPP-IV

A single dose of HWEC (250 mg/5 ml/kg; p.o) produced a significant ($n = 6$; $P < 0.05$–0.01) improvement in glucose tolerance and plasma insulin concentration compared to normal control rats (Figure 3B and C). This was associated with time-dependent inhibition of circulating DPP-IV ($P < 0.05$–0.001; Figure 3D). The area under curve showed 18% ($P < 0.05$) decline in DPP-IV activity (Figure 3D) compared with the high-fat-fed diet control rats. Positive controls, sitagliptin and vildagliptin (10 µmol/5 ml/kg) decreased ($P < 0.001$) enzyme activity by 70–73% (Figure 3D).

HWEC and glucose homeostasis and metabolic parameters in high-fat-fed rats

High-fat-fed diet rats depicted increased food, energy and fluid intake, body weight, blood glucose and plasma insulin levels compared to the lean control (Figures 4A–F and 5D and E). Twice daily oral gavage of HWEC significantly decreased the fluid intake and blood glucose level by 9% and 19%, respectively and increased plasma insulin by 14% from day 6 onwards compared to obese control

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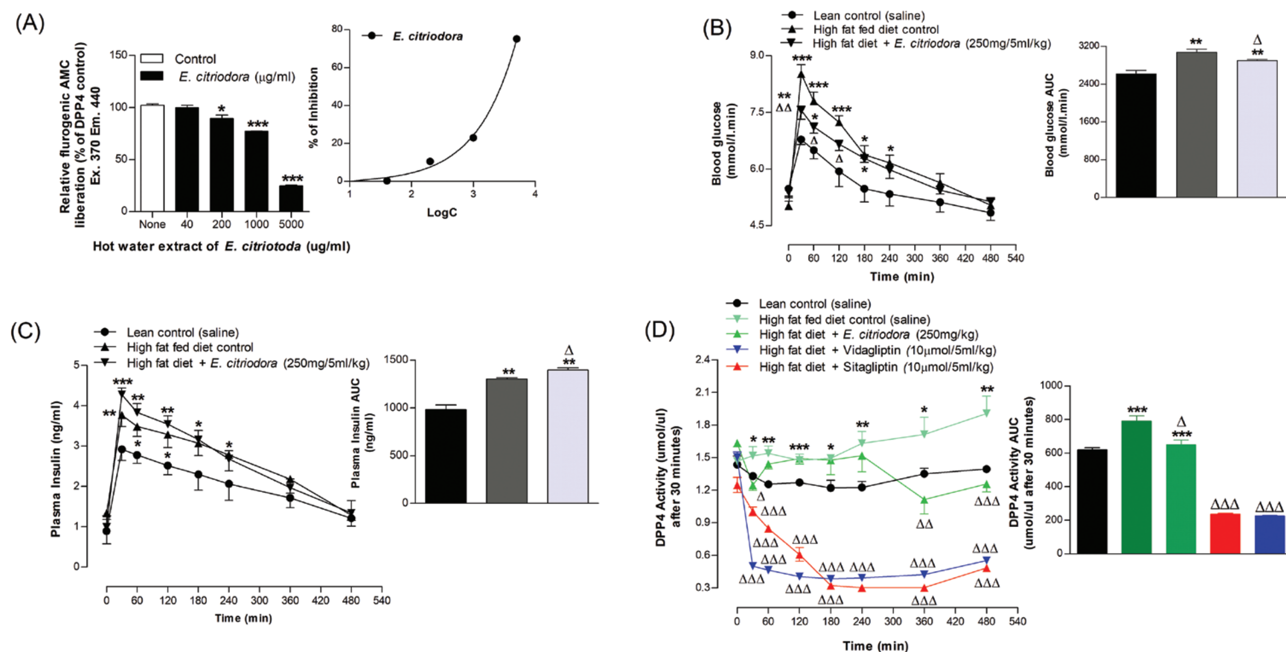

Figure 3 Effects of HWEC on (A) DPP-IV enzyme *in vitro*, (B) glucose tolerance, (C) plasma insulin and (D) plasma DPP-IV in high-fat-fed rats. *In vivo* parameters were evaluated before and after oral gavage of glucose alone (18 mmol/kg body weight, control) or with *E. citriodora* extract (250 mg/5 ml/kg body weight). Values for $n = 3$ for *in vitro* DPP-IV enzyme activity and $n = 6$ for parameters *in vivo* are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared to lean control and $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ and $^{\Delta\Delta\Delta}P < 0.001$ compared to high-fat-fed diet control rats.

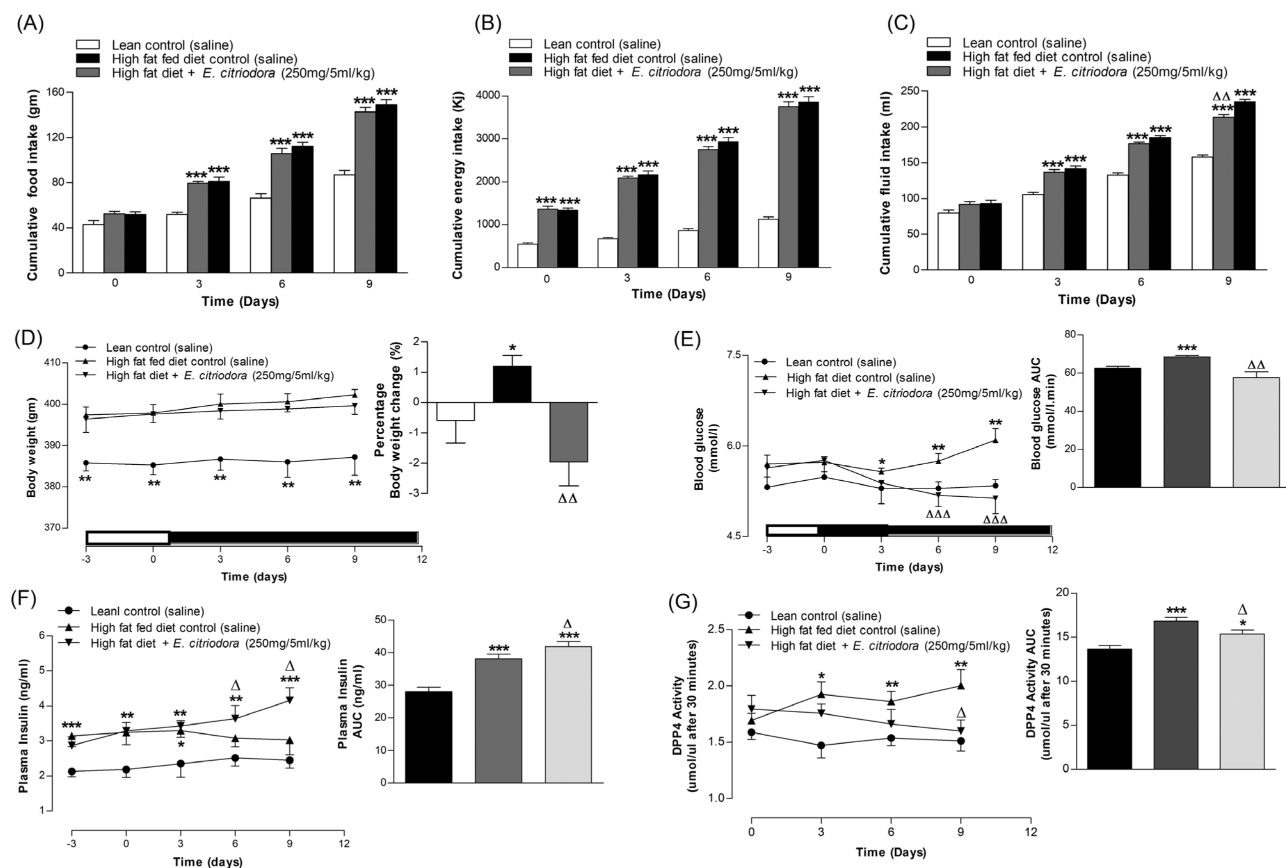

Figure 4 Effects of 9 days treatment of twice daily oral gavage of HWEC on (A) food intake, (B) energy intake, (C) fluid intake, (D) body weight, (E) blood glucose, (F) plasma insulin and (G) DPP-IV in high-fat-fed rats. Parameters were assessed twice a day, before and after oral gavage of *E. citriodora* leaves (250 mg/5 ml/kg, body weight). Values for $n = 8$ rats are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with lean control rats. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ and $^{\Delta\Delta\Delta}P < 0.001$ compared to obese control rats at the following time points.

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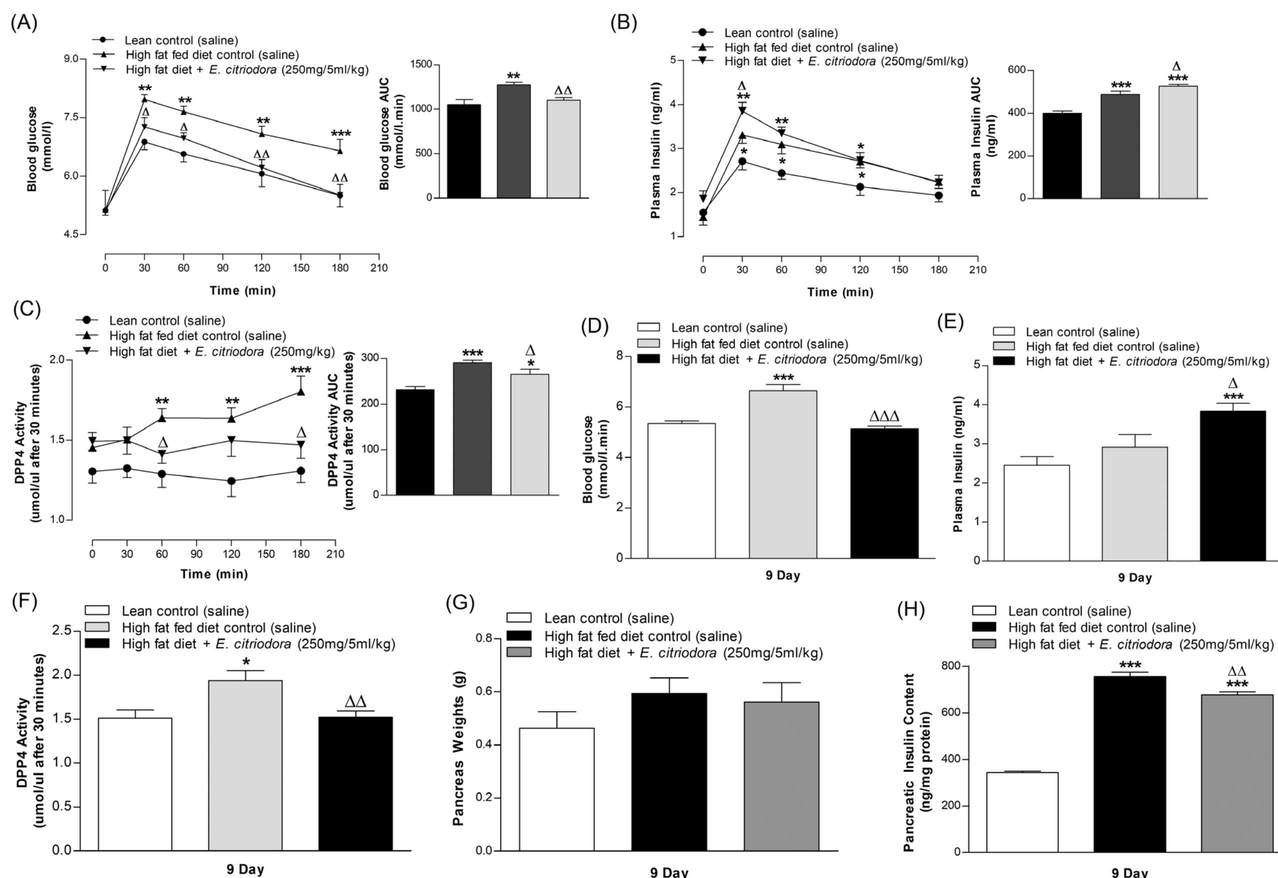

Figure 5 Long-term effects of twice daily oral gavage of HWEC leaves on (A) glucose tolerance, (B) plasma insulin and (C) plasma DPP-IV on day 6 and (D) blood glucose, (E) plasma insulin, (F) plasma DPP-IV, (G) pancreas weight and (H) pancreatic insulin content on day 9 in high-fat-fed rats. Parameters after 6 or 9 days of therapy with twice daily oral gavage of *E. citriodora* leaf (250 mg/5 ml/kg body weight) were measured. Values for $n = 8$ rats are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to lean control. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ and $^{\Delta\Delta\Delta}P < 0.001$ compared to obese control rats at the specific time points.

rats (P < 0.05–0.001; Figures 4C,E,F and 5D and E). No significant changes were observed in food, energy intake and body weight (Figure 4A,B,D). Hot water extract of *E. citriodora* also decreased plasma DPP-IV level in obese rats (P < 0.05) by 21% (Figures 4G and 5F).

HWEC and chronic glucose tolerance and plasma DPP-IV

HWEC (250 mg/5 ml/kg; p.o.) substantially (P < 0.05–0.01) enhanced glucose tolerance (at 30, 60, 120 and 180 min) after 6 days of therapy to high-fat-fed rats (Figure 5A). Increased plasma insulin concentrations (Figure 5B) and a 15–17% decrease in plasma DPP-IV enzyme activity were also consistent with this effect (P < 0.05; Figure 5C).

HWEC and pancreatic insulin content and islet size distribution and area

No significant changes in the pancreas weights of treated or untreated high-fat-fed or normal control rats were observed (Figure 5G). However, pancreatic insulin content significantly (54%; P < 0.001) increased in high-fat-fed rats compared to lean rats. Hot water extract of *E. citriodora* significantly (P < 0.01) decreased pancreatic insulin compared to high-fat-fed control rats (Figure 5H).

Figure 6 provides comparative pictures of pancreatic tissues of normal control (A), high-fat fed (B) and high-fat fed receiving HWEC (250 mg/5 ml/kg) (C), respectively. *Eucalyptus citriodora* resulted in a significant decrease in islets number per mm^2 in pancreas (Figure 6J). A significant (P < 0.001) decrease in islet area was also observed (P < 0.05; Figure 6D), but there were no changes in β-cell and α-cell areas compared to obese rats (Figure 6E and F). High-fat-fed rats, on the other hand, showed substantial increases in islet, β-cell and α-cell areas compared to lean control rats (Figure 6D,E,F). The numbers of large and medium sized islets were greater in high-fat-fed groups compared to lean controls (Figure 6G). The percentage of β-cells was also increased in high-fat-fed groups, whereas α-cell percentage was decreased (Figure 6H and I).

Insulin-releasing activity and structural identification of purified extract

RP-HPLC and LC-MS were used to isolate and characterize phytoconstituents from the crude leaf extract (Figures 7A and 8A–E). Five major fractions of *E. citriodora* were assayed for insulin-releasing activity using BRIN-BD11 cells (Figure 7B; peak samples (P-1 to P-5). It was observed that P-1 to P-5 greatly (P < 0.001) induced insulin secretion, but P-1 showed cytotoxicity assessed by cellular leakage of LDH (data not shown). Positive control, alanine (10 mM) was used (Figure 7B). Peak fractions of interest were further analysed using LC-MS for determination of molecular mass

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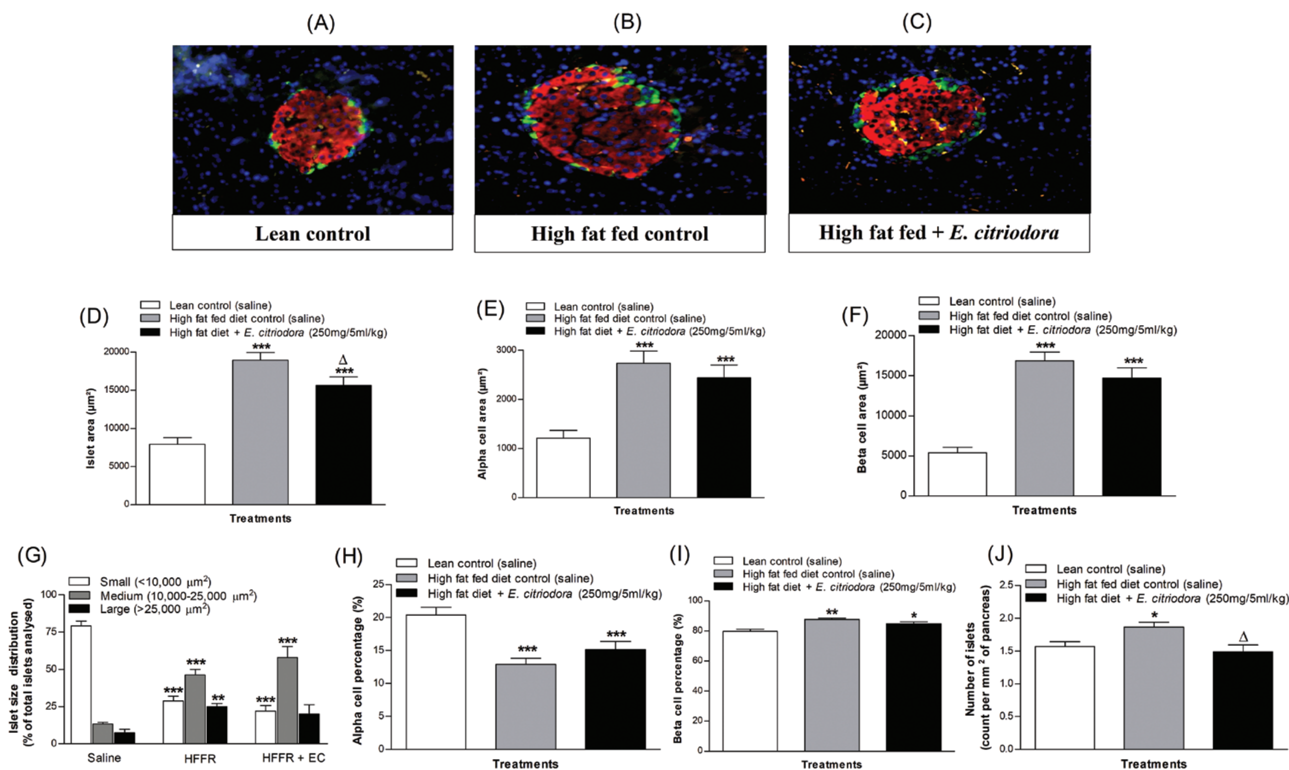

Figure 6 Effects of 9 days treatment of HWEC on islet morphology in high-fat-fed rats. Illustrative pictures of (A) lean control, (B) high-fat-fed control and (C) high-fat fed plus hot water extract of *E. citriodora* (250 mg/5 ml/kg) in rats presenting insulin in red, glucagon in green and DAPI in blue, (D) islet area, (E) α-cell area, (F) β-cell area, (G) islet size distribution, (H) percentage of α-cell, (I) percentage of β-cell and (J) number of islets (per mm²), respectively. Values for $n = 8$ are mean ± SEM (~50 islets per group). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to normal (Lean) control rats. $^{\Delta}P < 0.05$ compared to obese rats alone.

(Figure 8A–E). The molecular masses obtained for the three compounds corresponded with the previously isolated phytochemicals, quercitrin,[34] isoquercitrin[35] and rhodomyrtosone E.[17] The chemical structures and molecular masses of these three compounds are shown in Figure 9A–C.

Discussion

Type 2 diabetes and obesity are chronic disorders that are intricately linked such that body mass index is a useful marker for manifestation of insulin resistance and glucose tolerance.[2] Obesity is also associated with elevations of circulating free fatty acids, cytokines and other mediators of cellular damage that promote insulin resistance and impaired β-cell function on the pathway to diabetes development.[36] Previous studies have reported that *E. citriodora* possesses properties useful for the treatment of diabetes in folk medicine, but the mechanisms mediating such actions are unknown.[10, 17] This study was designed to investigate possible insulinotropic effects and other antihyperglycaemic actions of *E. citriodora*.

Eucalyptus citriodora leaves stimulated both glucose and concentration-dependent insulin release from BRIN-BD11 cells and isolated mouse pancreatic islets. To understand the molecular mechanism involved, the effects of non-toxic concentrations of *E. citriodora* were assessed in the presence or absence of modulators of β-cell function. This revealed inhibitory effects of diazoxide, verapamil and extracellular Ca^{2+} depletion, suggesting involvement of K_{ATP} channels, L-type calcium channels and Ca^{2+} influx in the insulin secretory actions Consistent with this view, the extract depolarized β-cell plasma membrane and increased intracellular Ca^{2+}. Since stimulatory effects persisted in β-cells depolarized by the sulphonylurea K_{ATP} channel blocker, tolbutamide or 30 mM KCl, additional actions must be triggered through K_{ATP}-independent pathways involving PI_3 (phosphatidylinositol) or adenylate cyclase/cAMP.[37] Indeed, insulinotropic effects of the extract were substantially potentiated by the phosphodiesterase inhibitor, IBMX. Interestingly, previous studies revealed the potential of *E. citriodora* in asthma as adjunct therapy,[38] with benefits ascribed to the elevation of cAMP in lung tissues thereby blocking replication in bronchial smooth muscle cells and promoting airway relaxation.[39]

Insulin primarily targets skeletal muscle and adipose tissue for post-prandial glucose regulation and defects in the signal transduction pathway in these cells with decreased GLUT-4 translocation is an important contributor to insulin resistance.[40, 41] In this study, effects of *E. citriodora* on glucose uptake were investigated using 3T3L1 differentiated adipocyte cells. The extract significantly stimulated glucose transport in the absence and presence of insulin. Previous studies have reported that phytomolecules such as gallic acid, egallic acid, berberine, quercetin and isoquercitrin[42, 43] may increase the glucose uptake by modulating AMPK activity. As *E. citriodora* extract has been reported to contain polyphenols like gallic acid and ellagic acid, these components might trigger this and other signalling pathways to improve glucose uptake.

Protein glycation is a physiological process that plays a significant role in the pathophysiology of diabetes.[44] The extract significantly inhibited insulin glycation in dose-dependent manner. Previous studies reported *E. citriodora* as potential antioxidant plant,[45] which may explain its ability to ameliorate protein damage induced by oxidative stress and inhibit insulin glycation.[46] Glycation of insulin

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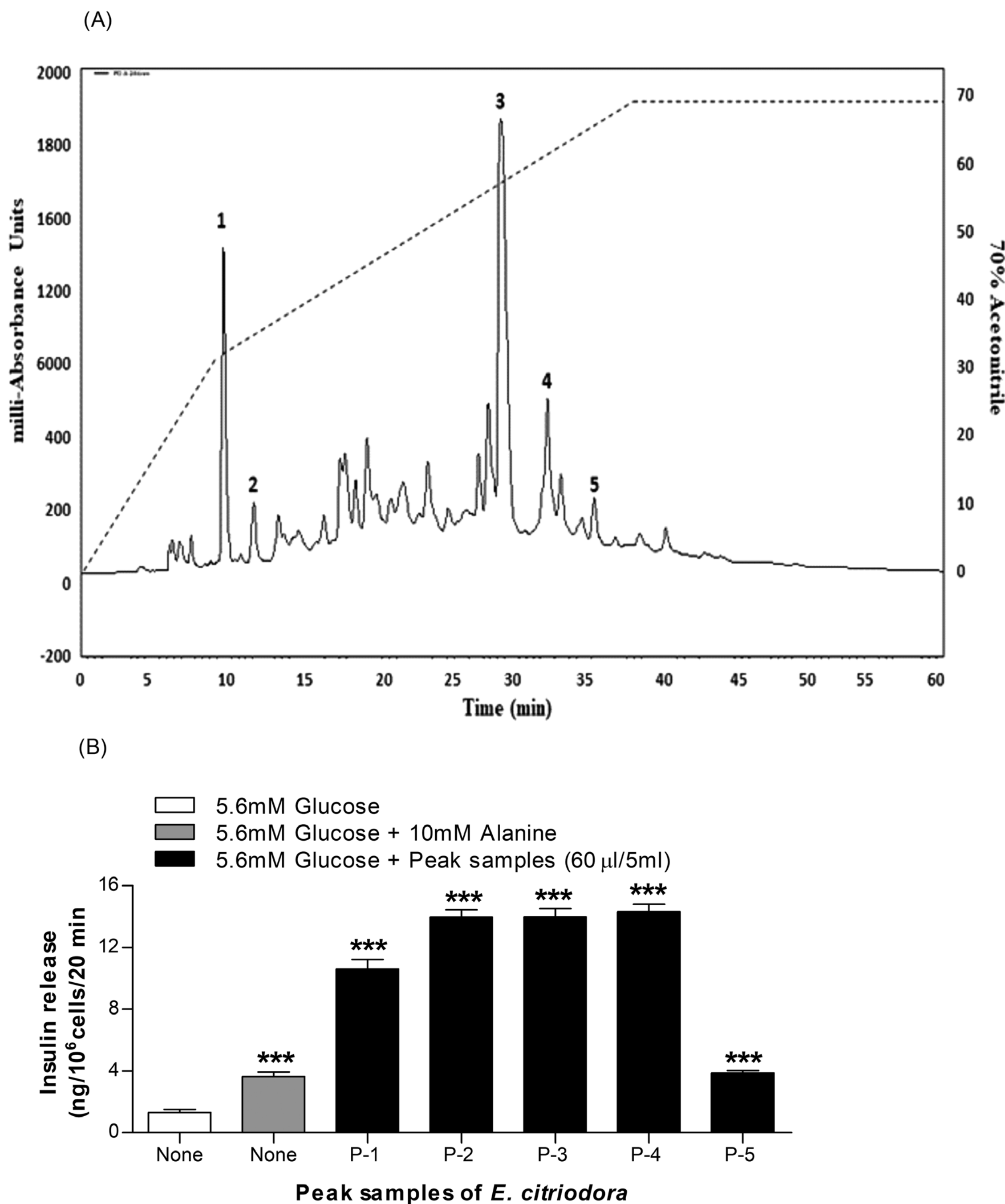

Figure 7 Representative (A) HPLC profile and (B) insulin-releasing effects of peak samples (1–5) of *E. citriodora* leaves. Crude extract was chromatographed at a flow rate of 1.0 ml/min on a (10 × 250 mm) semi-preparative 5 μm C-18 column (Phenomenex, UK). Using linear gradients of acetonitrile (0–20% up to 10 min, 20–70% up to 40 min), the concentration of the eluting solvent was increased. Compounds were detected by measurement of absorbance at 254–360 nm. Peak fractions 1–5 were collected and insulin-releasing activity assessed using BRIN-BD11 cells. Values for $n = 8$ rats are mean ± SEM for insulin release. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control.

results in an approximately 20% decrease in biological activity,[47] but significance of inhibition of protein glycation generally extends to countering adverse modification of functional and structural proteins involved in β-cell dysfunction and diabetic complications.

A useful approach to management of post-prandial hyperglycaemia is inhibition of the digestive enzymes, α-amylase and α-glucosidase. This slows entry of glucose into the blood, thereby moderating the glycaemic excursion and demands on β-cells following feeding. *E. citriodora* extract inhibited starch breakdown in a concentration-dependent manner which might reflect presence of flavonoids which have been reported to inhibit activity of these enzymes.[48] Decrease of gastrointestinal absorption and glucose diffusion is another important means of moderating glucose response to feeding.[29] In this study, a simple *in vitro* dialysis method was used to mimic the gut barrier and investigate the effects of *E. citriodora* on glucose diffusion. Extracts of *E. citriodora* exerted significant

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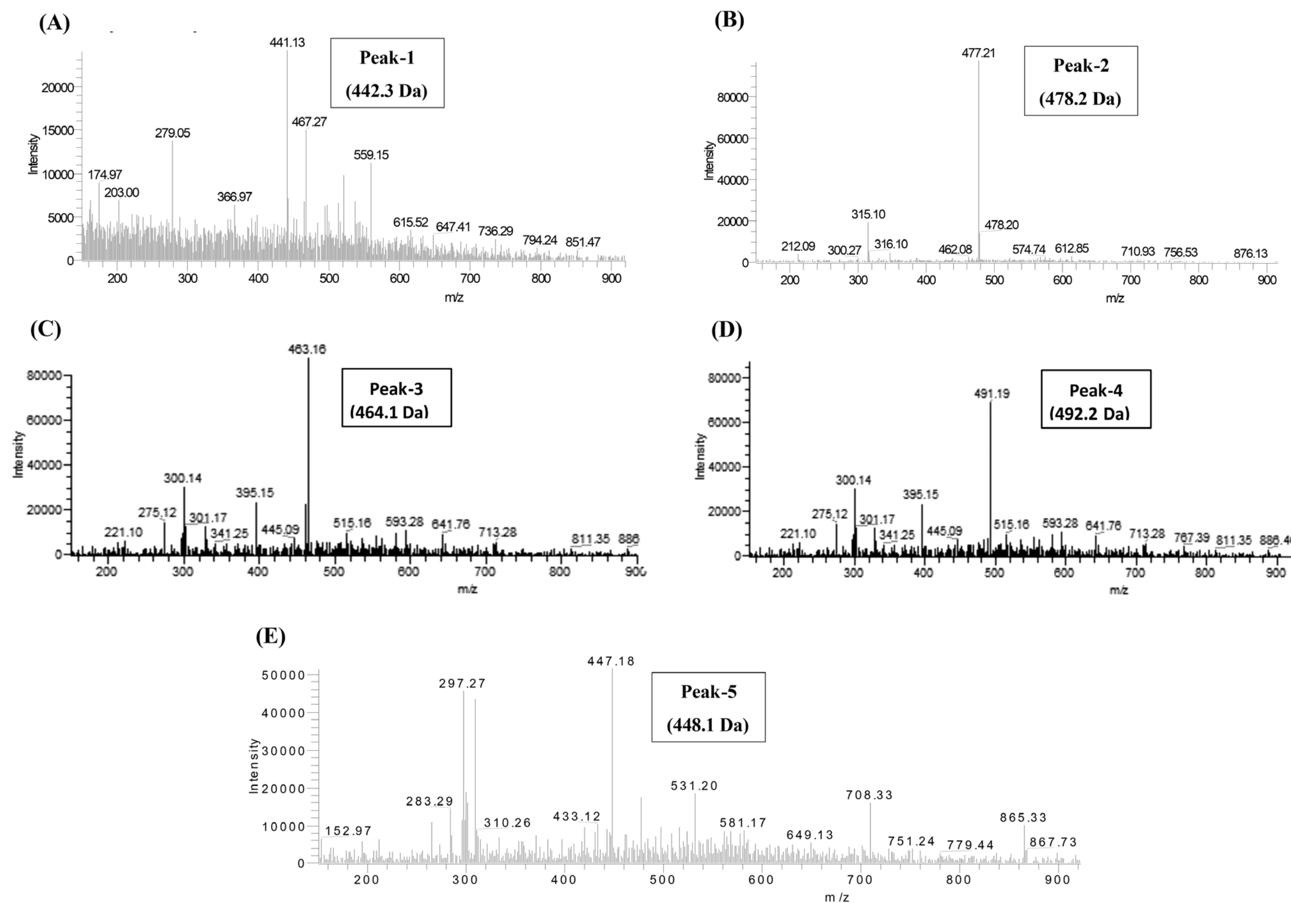

Figure 8 Molecular masses of insulin-releasing peak samples of *E. citriodora* leaves by LC-MS analysis. A Kinetex 5µm F5 LC column ((150 × 4.6 mm; Phenomenex) was used to isolate Peaks 1–5 on a Spectra System LC. The ratio of mass to charge (m/z) versus peak intensity was calculated at a wavelength of 220–360 nm.

concentration-dependent inhibitory effects on glucose movement into an external solution through dialysis membrane. Decreased hyperglycaemia of STZ-induced diabetic animals induced by dietary supplements of *E. citriodora* was attributed to the modulation of glucose movement from the gut.[38] Several lines of evidence also suggest that the viscosity and presence of soluble dietary fibres retard glucose transport by slowing transit time and modifying both the digestive and absorptive processes.[29, 49]

Sprague-Dawley rats fed with high-fat diet replicate obesity and metabolic disorders such as tolerance to insulin reminiscent of type 2 diabetes.[50, 51] In an acute *in vivo* experiment, *E. citriodora* extract significantly improved glucose tolerance and plasma insulin responses of obese rats. These results were reinforced by results of further long-term studies in obese rats that showed remarkable improvements induced in blood glucose, glucose tolerance and circulating insulin compared with obese control rats. As well as improving β-cell function directly, the extract also inhibited activity of DPP-IV both *in vitro* and *in vivo*. This enzyme deactivates incretin hormones by cleavage of N-terminal that leads to generation GLP-1 (9–36) and GIP (3–42).[52] Accordingly, *E. citriodora* may also indirectly improve β-cell function by increasing the half-life and stimulatory effects of endogenous GLP-1 and GIP. Interestingly, previous studies have reported that various plants and constituent phytochemicals possess DPP-IV inhibitory activity.[53, 54]

Insulin resistance due to consumption of a high-fat-fed diet, reduces insulin-mediated glucose uptake by the peripheral tissues, causes unrestrained hepatic glucose output and thereby increases endogenous insulin demand.[55] This causes β-cells to hypersecrete and expand β-cell mass due to proliferative activity.[56] In this study, high-fat-fed rats showed a significant increase in islet, β-cell and α-cell areas which were partly normalized by *E. citriodora*. Significant changes were noted in both the area and total number of islets per mm^2. The increase in pancreatic insulin content observed with *E. citriodora* might therefore be mediated by modulation of β-cells mass as well as direct actions and possible secondary effects mediated by endogenous GLP-1 and GIP.[22]

Eucalyptus citriodora extract was analysed for the identification and characterization of active compounds targeting insulin secreting β-cells. Fractions were collected from the RP-HPLC and five major peaks were tested using BRIN-BD11 cells. All peaks significantly stimulated insulin release (P < 0.001), but P-1 was associated with LDH release and cellular toxicity (data not shown). This evaluation of bioactivity was followed by LCMS to identify molecular mass of the active RP-HPLC fractions. Molecular masses of three compounds isolated corresponded with those of quercitrin, isoquercitrin and rhodomyrtosone E.[17, 34, 35] This result is in line with previous findings reporting that *E. citriodora* contained isoquercitrin and rhodomyrtosone E.[17] No previous studies have validated quercitrin as a phytoconstituents of *E. citriodora*, but others have noted that quercitrin is a glycoside derived from quercetin and deoxy sugar rhamnose.[57] The study by Wang *et al.*[17] is particularly noteworthy because, as well as rhodomyrtosone E, it reported 12 other compounds in *E. citriodora* including seven triterpenes, four flavonoids and one polyphenolic compound, gallic acid. Several of these may contribute to antidiabetic activity.[58]

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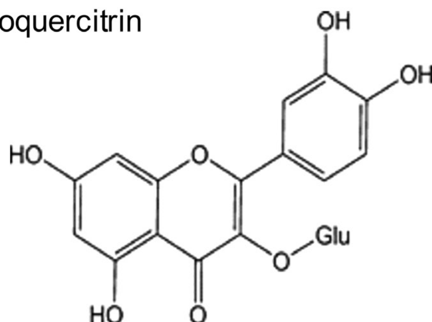
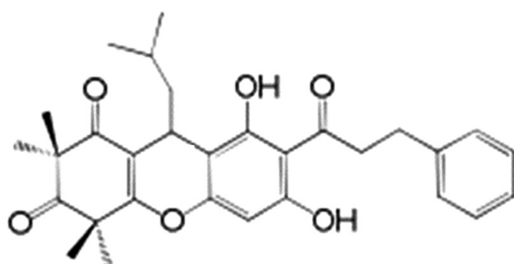
(A) Isoquercitrin



(B) Rhodomyrtosone E



(C) Quercetin-3-rhamnoside

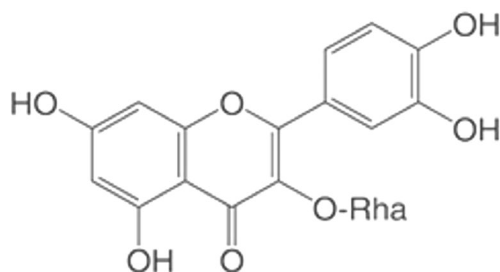


Figure 9 Chemical structure of compounds isolated from *E. citriodora* leaves. Chemical structure of flavonoids, corresponding to the molecular formulae: (A) $C_{21}H_{20}O_{12}$, (B) $C_{30}H_{34}O_{6}$ and (C) $C_{21}H_{20}O_{11}$.

Conclusion

This study has shown that *E. citriodora* leaf extract exerts significant antidiabetic effects *in vitro* and in high-fat-fed rats due to a variety of pancreatic and extra pancreatic actions. These include stimulation of both the secretion and action of insulin as well as inhibition of DPP-IV activity, starch digestion, glucose diffusion and protein glycation. Phytochemicals acting at level of the β-cells are likely to include quercitrin, isoquercitrin and rhodomyrtosone E, possibly with their metabolites Further investigations are warranted to fully characterize the benefits of *E. citriodora* and its phytoconstituents for the potential prevention, treatment and management of type 2 diabetes in man.

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Author Contributions

P.R.F. and Y.H.A. designed the project and contributed equally in supervision; P.A. conducted the study, analysed the data and interpreted the findings; P.A. and P.H. also characterized phytochemicals; P.A. and P.R.F. drafted the manuscript; the revised manuscript was edited by P.A. and P.R.F.; the final version was authorized by all contributors.

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Conflict of Interest

The researchers declare that this manuscript is not associated with duality of concern.

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