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Research Paper

In vitro and *in vivo* antihyperglycemic activity of the ethanol extract of *Heritiera fomes* bark and characterization of pharmacologically active phytomolecules

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Abstract

Objective This study aimed to demonstrate the mechanistic basis of *Heritiera fomes*, which has traditionally been used to treat diabetes.

Methods Clonal pancreatic β-cells and primary islets were used to measure insulin release. 3T3-L1 cells were used to analyse insulin action, and *in vitro* systems were used to measure further glucose-lowering activity. *In vivo* assessment was performed on streptozotocin (STZ)-induced type-2 diabetic rats and reversed-phase-HPLC followed by liquid chromatography mass spectrometry (LC-MS) to detect bioactive molecules.

Key findings Ethanol extract of *Heritiera fomes* (EEHF) significantly increased insulin release with stimulatory effects comparable to 1 μM glucagon-like peptide 1, which were somewhat reduced by diazoxide, verapamil and calcium-free conditions. Insulin release was stimulated by tolbutamide, isobutyl methylxanthine and KCl. EEHF induced membrane depolarization and increased intracellular Ca^{2+} levels. EEHF enhanced glucose uptake in 3T3L1 cells and decreased protein glycation. EEHF significantly inhibited postprandial hyperglycaemia following sucrose loading and inversely elevated unabsorbed sucrose concentration in the gut. It suppressed glucose absorption during *in situ* gut perfusion. Furthermore, EEHF improved glucose tolerance, plasma insulin and gut motility, and decreased plasma dipeptidyl peptidase IV activity. Procyanidins, epicatechin and proanthocyanidins were some of the identified bioactive constituents that may involve in β-cell actions.

Conclusions This study provides some evidence to support the use of *H. fomes* as an antidiabetic traditional remedy.

Keywords: diabetes mellitus; *Heritiera fomes*; DPP-IV; glucose; insulin

Introduction

Diabetes mellitus (DM), which occurs as a result of excessive blood sugar levels, is one of the main metabolic syndromes. Based on its aetiology and clinical presentation, DM is classified as either type-1, type-2, gestational or other specific types. Type-1 diabetes is the consequence of the almost complete destruction of pancreatic β-cells, whereas type-2 diabetes develops in the case of β-cell failure and/or insulin resistance.[1] Type-2 diabetes mellitus (T2DM) is currently the most dominant type worldwide, notably due to the rise in obesity cases.[2, 3] Chronic disorders such as DM impact many different body organs, and diabetic patients have a high risk of heart attacks and strokes.[2] Diabetes is also the leading cause of kidney failure, neuropathy and blindness.[4]

T2DM treatment involves diet control, exercise and the use of one or more oral or injectable antihyperglycemic drugs. The latter include SGLT-2 inhibitors, insulin sensitizers/secretagogues and dipeptidyl peptidase IV (DPP-IV) inhibitors.[5] One of the major drawbacks of such agents, however, is that they lead to weight gain, gastrointestinal disturbances, kidney disorders and hypoglycaemia.[6] In this context, it is time to discover novel antidiabetic agents.

Plants have a long history of being used medicinally, and numerous studies have demonstrated the biological potential of plant extracts/phytochemicals. Relatively few plants, however, have been scientifically explored for their antidiabetic activity. A mangrove evergreen tree named *Heritiera fomes* is commonly known as the Sunder/Sundari tree throughout Asia and especially in the Sundarban region of Bangladesh. The traditional medicine practitioner used *H. fomes* to treat several disorders such as diarrhoea, constipation, indigestion and dysentery.[7] *Heritiera fomes* leaves and seeds are also consumed as edible parts of the plant in many regions such as the Andaman Islands.[8] In rural areas, the bark is used for diabetes and goitre.[9]

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Previous studies have revealed that *H. fomes* is rich in procyanidins, which have been found to have beneficial effects in preventing obesity and treating T2DM.[10] Other studies on the bark and leaves have demonstrated antidiabetic and cardioprotective activity.[11, 12] The plant also has remarkable antioxidant,[9] antinociceptive, antimicrobial[13] and anticancer activity.[14] However, the specific mechanisms behind the glucose-lowering effects remain uncertain. We anticipated that possible phytoconstituents that appear in *H. fomes* may exhibit a range of antidiabetic properties. Therefore, this study aimed to evaluate the *in vitro* and *in vivo* antihyperglycemic activity of an ethanolic extract of *H. fomes* bark.

Materials and Methods

Plant extract collection and preparation

Heritiera fomes bark was collected from Sundarbans, Bangladesh. A botanist confirmed the plant's identity and issued a herbarium number 43206. The barks of *H. fomes* were thoroughly washed and air-dried at 40°C in oven followed by ethanolic extraction. The dried powdered bark (200 g) was extracted with 1-litre ethanol (80%, v/v). The mixture was kept on a shaker at 900 *g* for 3 to 4 days at 4–8°C. The extract was concentrated to dryness under reduced pressure at <40°C after filtration using Whatman no. 1 filter paper. The resulting ethanol extract of *Heritiera fomes* (EEHF) was dried, and the final residue (~5 g) was stored at 4°C for further experiments.[15] We used dimethyl sulfoxide to dissolve the freeze-dried powder to treat cells.

Insulin-release assessments

Clonal pancreatic β-cells (BRIN-BD11) and primary (mouse) islets were used to determine the insulin-releasing action of EEHF.[16] The islets were isolated from mice pancreas using collagenase P from *Clostridium histolyticum* and cultured for 24–48 h at 37°C[17] BRIN-BD11 cells and the islets were incubated at 37°C with EEHF with/without insulin secretagogues at different glucose concentrations (1.1, 5.6 or 16.7 mM). After 20 min of incubation, the supernatants were collected, centrifuged and stored at –20°C before insulin radioimmunoassay.[15, 18] Glucagon-like peptide 1 (GLP-1) (10^{-6} & 10^{-8} M) and alanine (10 mM), which promote insulin release, were used as positive controls.

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

A FLIPR MP and ($[Ca^{2+}]_i$ *5* assay kit (Molecular Devices, Sunnyvale, CA, USA) was used to investigate the impact of EEHF on the membrane potential (MP) and $[Ca^{2+}]_i$ levels. Changes in signal intensity due to the addition of EEHF were monitored using a fluorometric assay.[15]

Cellular glucose uptake

3T3L1 differentiated cells were used to determine the glucose uptake effects of EEHF. Cells with/without 100 nM insulin were treated with 50 µL of a stock solution of EEHF (200 µg/ml) and then incubated at 37°C for 30 min. After 5 min of addition of the fluorescent glucose analogue 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose (50 nM), the mixture was washed with ice-cold phosphate buffered saline. Coverslips were fitted on the slides, and the corners were sealed with nail polish. Images were captured from each coverslip of the four corners with a microscope at ×10 magnification to dictate the fluorescence intensity.[19]

Insulin glycation

The impact of EEHF on the glycation of insulin was evaluated according to a previously described methodology.[20] Mixtures of D-glucose (246.5 mM), human insulin (1 mg/ml), sodium phosphate buffer (10 mM, pH 7.4) and $NaBH_3CN$ (0.0853 g/ml) were incubated at 37°C for 24 h with different doses of EEHF (50–200 µg/ml). The absence of EEHF in the mixture was used as a control. The mixture (total 1 ml) had a pH of 7.4. After 24 h incubation, the reaction was brought to end by adding 30 µL of 0.5 M acetic acid. Reversed-phase (RP)-HPLC was used to assess glycated and non-glycated insulin levels.[15]

DPP-IV enzymatic activity

The previously described method was applied to analyse the changes in DPP-IV enzymatic activity.[21] Reagents including DPP-IV enzyme (8 mU/ml) and Gly-Pro-AMC (200 µM) substrate were incubated in microplates of 96-well (Greiner) with/without EEHF. A Flex Station 3 (Molecular Device) (2.5 nm slit width) was used to measure fluorescence intensity.[18]

Starch digestion

The impact of EEHF on digestive enzymes was evaluated using previously described methodologies.[22] Briefly, a solution was made by adding starch (100 mg) in distilled water (3 ml) in the presence/absence of EEHF (62.5–1000 µg/ml) and heat-stable α-amylase (40 µl of 0.01%) (Sigma-Aldrich, Saint Louis, Missouri, USA). The mixtures were incubated for 20 min at 80°C. The diluted mixtures were re-incubated with 30 µl of amyloglucosidase (0.1%) (Sigma-Aldrich, Saint Louis, Missouri, USA) at 60°C for 30 min. Following the final incubation, samples were collected and stored at 4°C until further analysis using the liquid glucose oxidase-phenol 4 aminophenazone (GOD-PAP) method (Randox GL 2623).[15] A positive control, α-glucosidase inhibitor, acarbose (1 mg/ml) was used.

Glucose diffusion

On a cellulose ester dialysis tube, an *in vitro* glucose diffusion test was performed.[23] The tube was filled with 2 ml of 0.9% NaCl and 220 mM glucose with (treatment) or without (control) EEHF (0.2–25 mg/ml). The samples were loaded on an orbital shaker at 500 *g* at 37°C and collected after 24 h. Glucose liberation was analysed outside the tube as previously described.[15]

Animals

Male Long-Evans rats (Envigo UK, 150–200 g) were kept under an ideal temperature of 22 ± 5°C, humidity of 55–65 % and a 12-h light/dark cycle. The rats were individually housed and supplied with proper diet and water ad libitum.[18] Streptozotocin (STZ) [90 mg/kg body weight (b.w.)] was injected as a single intraperitoneal dose in 150 newborn rats (2 days of age) to later induce T2DM.[24] The rats were monitored for 3 months following the administration of STZ, and those that exhibited a blood glucose level of 8–12 mmol/l after an

oral glucose tolerance test (OGTT; 2.5 g/kg, b.w.) were selected as T2DM rats for further experiments.[25] Approximately, 146 rats developed T2DM after 12 weeks of age, whereas 144 of them were used in the experiments. There were no fatalities, and by 14 weeks of age, all STZ-induced rats had developed diabetes signs.[18] The experimental protocol was followed as per the 'Principles of Laboratory Animal Care' (NIH publication No. 85-23, revised 1985) in compliance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63EU for animal experiments. All essential actions were taken to prevent any animal suffering. All experiments were carried out following the methods approved by the Animal Welfare and Ethical Review Board (AWERB) (10 July 2017) at Ulster University and were maintained by the PIL1822 and PPL 2804 UK Home Office Animal project/personal license numbers, which were approved on 06 May 2016 and 05 February 2017, respectively.

Residual gut sucrose content

The residual gut sucrose content was measured as described previously.[26] A bolus dose of sucrose (2.5 g/5 ml/kg b.w.) was orally gavaged to 24 h-fasted T2DM rats with/without EEHF treatment (250 mg/5 ml/kg, b.w.). The animals were euthanized for gut sucrose residual content analysis. The whole gastrointestinal (GI) tract was segmented into six different fragments as shown in Figure 4A–F. Each fragment was cleansed in acidified ice-cold saline (0.9% NaCl) and then centrifuged at 3000 *g* for 10 min. The supernatants were collected, boiled for 2 h with concentrated H_2SO_4 to hydrolyse the sucrose content and then neutralized to pH 7.4 by adding NaOH (1 M). The residual gut sucrose content was measured from glucose concentration that was released from the GI tract.[27]

Intestinal glucose absorption

An *in situ* technique was applied to determine the intestinal perfusion of glucose. In this process, EEHF (5 mg/ml equal to 0.25 g/5 ml/kg) supplemented with Krebs Ringer Bicarbonate (KRB) buffer and glucose (54 g/l) was administered to 36 h-fasted T2DM rats. The mixture was perfused through the pylorus, and the perfusate was collected at the end of the ileum *via* a catheter. The control group received only the KRB buffer–glucose mixture. The mixtures were perfused at a constant rate (0.5 ml/min) for 30 min, and the amount of glucose in solution before and after the perfusion was calculated to determine the percentage of glucose absorbed.[25]

Gastrointestinal disaccharidase activity and motility

The intestinal disaccharidase (IDs) enzyme activity following the oral administration of EEHF (250 mg/5 ml/kg) to 24 h-fasted T2DM rats was assessed as previously described.[26] One hour after the administration, the rats were euthanized, and the small intestine was separated, segmented longitudinally and cleansed with ice-cold saline. The tissue was homogenized and diluted in saline to 10 ml. The homogenized aliquots were treated in a 40 mM sucrose solution for 1 h at 37°C. The IDs enzyme activity was assessed. As a positive control, acarbose (200 mg/5 ml/kg), a known IDs enzyme inhibitor, was used.

To check the GI motility, a barium sulfate ($BaSO_4$) milk solution was used as previously described.[28] Briefly, 12 h-starved STZ-induced type-2 diabetic rats were treated with EEHF (250 mg/5 ml/kg) orally and an hour later treated with a 10% $BaSO_4$ solution. The control group received only distilled water (10 ml/kg). After 15 min, the rats were sacrificed, and the length travelled by $BaSO_4$ through the small intestine was assessed and calculated as a percentage of the total length from the pylorus to the ileocecal junction. As a positive control, bisacodyl (1 mg/5 ml/kg b.w.) was utilized.

Purification of crude extract

A previously described purification method was used to isolate possible active compound/s from a bark extract.[15] The crude sample was dissolved in 0.12% (v/v) trifluoroacetic acid (TFA)/water, and the substances were separated according to affinity by RP-HPLC. At a flow rate of 1.0 ml/min, the column was equilibrated with 0.12% (v/v) TFA/water. The eluting solvent was maintained at a gradient ratio (acetonitrile concentration increased from 20% to 70% within the eluting solvent) for 40 min cycle.

Structural characterization

The liquid chromatography mass spectrometry (LC-MS) via electrospray ionization mass spectrometry (ESI-MS) technique was used for measuring molecular masses of purified samples (peaks) from RP-HPLC. As described before,[15] on a Spectra System LC (Thermo Finnigan, San Jose, California, USA), these samples were identified using a Kinetex 5m F5 LC column (150 × 4.6 mm, Phenomenex), and a UV detection system at 220–256 nm was used to detect peaks.

Statistical analysis

Graph Pad prism 5 was used to interpret statistical significance for all data, and it was depicted as mean ± SEM. To analyse data, an unpaired Student's *t*-test and one-way analysis of variance (ANOVA) were employed, where applicable. The hypothetical significance limit was $P < 0.05$.

Results

EEHF and insulin release

With 5.6 mM glucose, the basal insulin release from pancreatic β-cells was 1.30 ± 0.05 ng/10^6 cells/20 min. The positive control alanine (10 mM) led to an upsurge of the basal rate to 6.70 ± 0.90 ng/10^6 cells/20 min. Incubation of EEHF at concentrations ≥ 1.6 µg/ml with BRIN-BD11 cells potentially increased the basal insulin rate ($P < 0.05$–0.001) (Figure 1A and B). Likewise, the release of insulin was 1.85 ± 0.06 ng/10^6 cells/20 min at 16.7 mM glucose, it increased to 10.62 ± 0.35 ng/10^6 cells/20 min with KCl (30 mM) (Figure 1B). At 16.7 mM glucose, EEHF notably increased secretion of insulin ($P < 0.05$–0.001) at ≥8 µg/ml (Figure 1B). EEHF, at doses ranging from 1.6 to 1000 µg/ml, exerted a dose-dependent increase in the release of insulin without hampering cell feasibility ($P < 0.001$; Figure 1A and B). At high concentration, EEHF (5000 µg/ml) demonstrated greater potency (Figure 1A and B) but caused a significant increase in lactate dehydrogenase (LDH) release, resulting in cellular damage (Supplementary Figure S1A and B). Different concentrations of EEHF induced insulin secretion by primary islets with 16.7 mM glucose (Figure 1C). EEHF demonstrated a significant ($P < 0.05$–0.001) increase in insulin secretion at

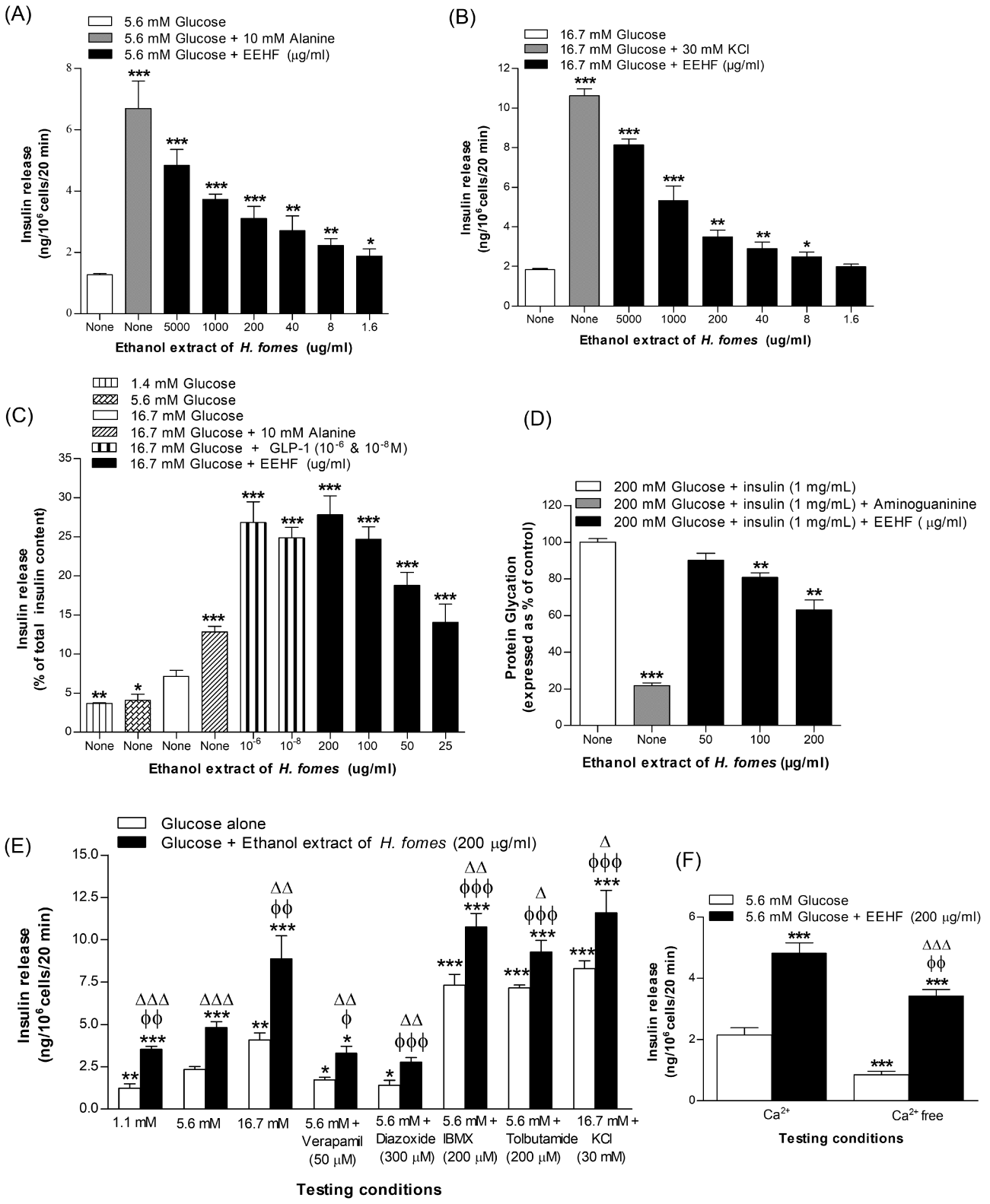

Figure 1 Effects of EEHF on (A & B) BRIN-BD11 cells insulin release, (C) primary islets insulin release, (D) glycation of insulin, (E) insulin release with/without stimulators/inhibitors and (F) without extracellular calcium. Values are mean ± SEM for $n = 4$–8 (insulin secretion) and $n = 3$ (insulin glycation). *, **, ***$P < 0.05$–0.001 versus controls; ϕ, ϕϕ, ϕϕϕ$P < 0.05$–0.001 versus 5.6 mM glucose with EEHF; Δ, ΔΔ, ΔΔΔ$P < 0.05$–0.001 versus incubations without EEHF.

doses ≥25 μg/ml. At high concentrations, the release of insulin exceeded that of the positive control GLP-1 (Figure 1C).

A dose (200 μg/ml) of EEHF induced secretion of insulin with stimulators. EEHF regulated stimulatory activity of insulin at 16.7 mM glucose. On the other hand, incubation with diazoxide (300 μM), a K^+ channel activator and verapamil (50 μM), a blocker of L-type voltage-dependent Ca^{2+} channels, decreased insulin secretion by 32–43% (Figure

1E). In KCl (30 mM)-generated membrane depolarized cells, EEHF increased insulin release by 1.4-fold, and this was synergized in the presence of isobutyl methylxanthine (IBMX; $P < 0.001$) and tolbutamide ($P < 0.001$) (Figure 1E). In the absence of extracellular calcium ions, insulin release plunged to 29% approximately ($P < 0.01$) (Figure 1F).

EEHF and MP and $([Ca^{2+}])_i$

In BRIN-BD11 cells, 30 mM KCl caused 85% depolarization (Figure 2A), while EEHF potently ($P < 0.001$) increased membrane depolarization and intracellular calcium ion levels by ~80% (Figure 2A and B). The intracellular calcium concentration increased to 95% with 10 mM alanine (Figure 2B).

EEHF and insulin glycation

A substantial ($P < 0.05$–0.01) decrease in insulin glycation was seen with EEHF (19–37% reduction at concentrations ≥100 μg/ml), while aminoguanidine (44 mM) decreased insulin glycation by approximately 85% (Figure 1D).

EEHF and glucose uptake and insulin action

Figure 2C–F illustrates the fluorescence intensity of EEHF in the presence/absence of 100 nM insulin. With 100 nM insulin, EEHF exhibited a 1.5-fold elevation in glucose uptake ($P < 0.05$), while EEHF alone incited a 1.2-fold rise in glucose uptake ($P < 0.05$). Insulin alone enhanced the uptake of glucose by 1.3-fold ($P < 0.01$; Figure 2G).

EEHF bark and starch digestion

At concentrations ≥250 μg/ml, EEHF potentially repressed glucose release from starch (Figure 2H). Acarbose (1 mg/ml) reduced the enzymatic activity by up to 91% (data not shown). Correspondingly, EEHF (1000 μg/ml) inhibited enzymatic activity by approximately 42% (Figure 2H).

EEHF and *in vitro* glucose diffusion

EEHF demonstrated a concentration-dependent (200–5000 μg/ml) significant reduction ($P < 0.05$–0.01) in glucose absorption over the 24-h incubation period. At a dose of 5 mg/ml, EEHF depleted glucose release up to 23% ($P < 0.01$; Figure 2I).

EEHF and *in vitro* DPP-IV enzymatic activity

EEHF demonstrated a 9–38% decrease in DPP-IV enzymatic activity in a concentration-dependent manner (40–5000 μg/ml) ($P < 0.01$–0.001; Figure 3A), while sitagliptin (1–10 000 nM) inhibited DPP-IV activity by up to 15–96% (data not shown).

EEHF and glucose tolerance, plasma insulin and DPP-IV enzyme level

EEHF (250 mg/5 ml/kg, b.w., given as a single dose) in combination with glucose (2.5 g/5 ml/kg, b.w.) improved glucose tolerance in different time points as shown in Figure

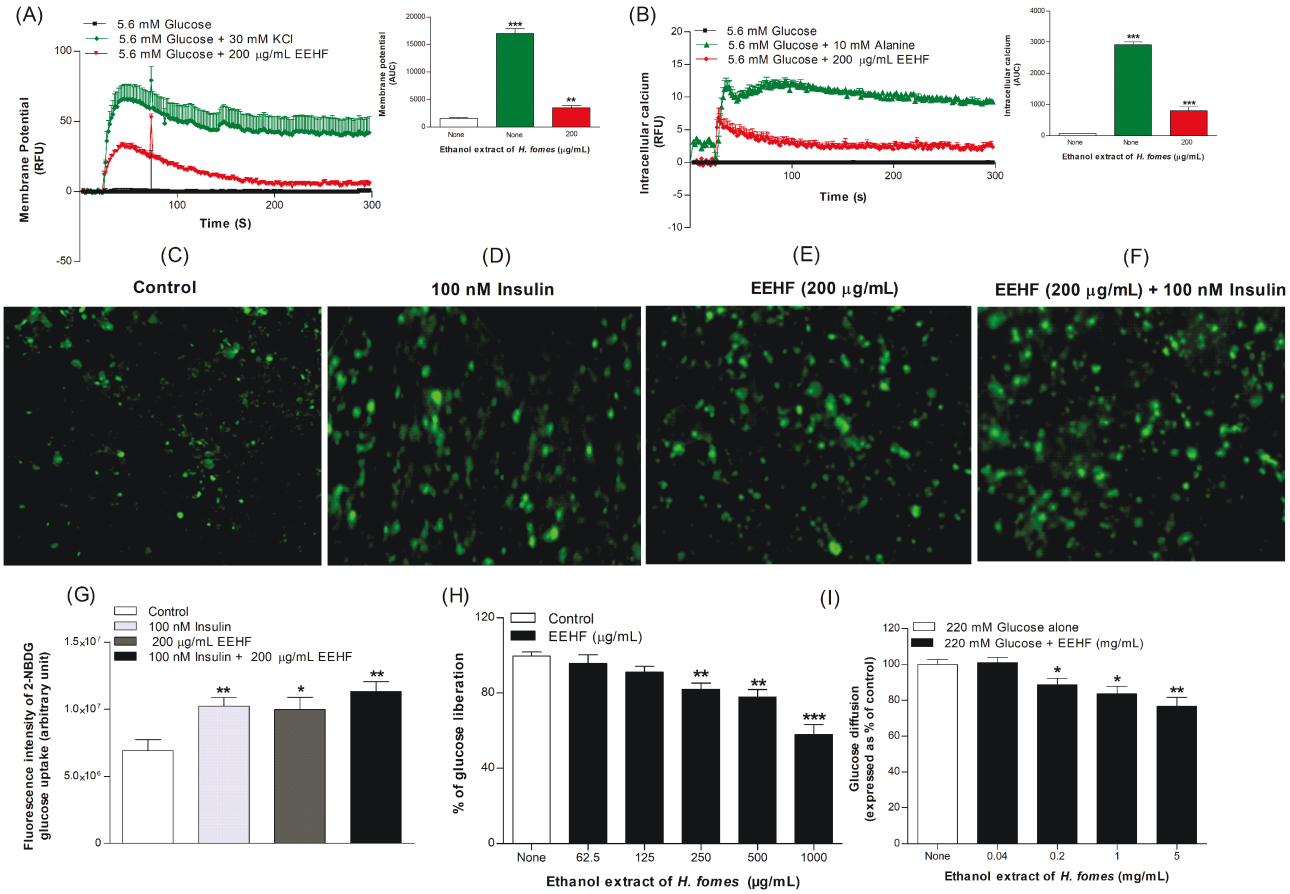

Figure 2 Effects of EEHF on (A) membrane depolarization and (B) cytoplasmic calcium in BRIN-BD11 cells, (C–G) uptake of glucose by 3T3L1 adipocytes, (H) *in vitro* starch digestion and (I) diffusion of glucose. Fluorescence intensity was monitored in cells incubated with EEHF without (E) or with (F) insulin (100 nM). Images are captured at a magnification of ×10. Values are mean ± SEM for $n = 4$ (uptake of glucose, starch digestion and diffusion of glucose) and $n = 6$ (depolarization of membrane and cytoplasmic calcium); *, **, ***$P < 0.05$–0.001 versus control.

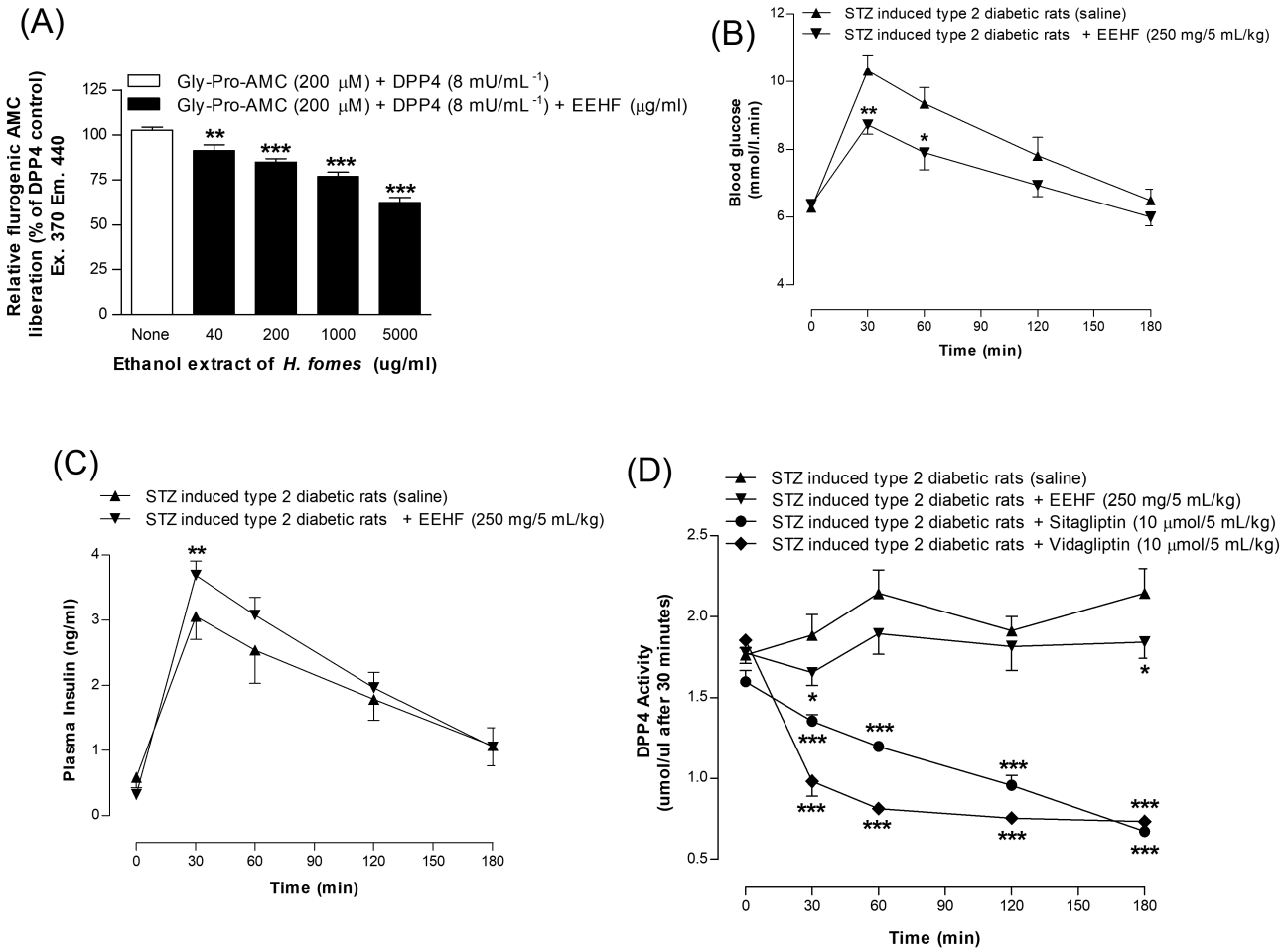

Figure 3 Acute effects of EEHF on (A) *in vitro* DPP-IV enzymatic activity, (B) oral glucose tolerance, (C) plasma insulin level and (D) DPP-IV enzymatic activity in STZ-induced type-2 diabetic rats. Parameters were assessed before and later oral gavage of 18 mmol glucose/5 ml/kg, body weight (control) with or without EEHF (250 mg/5 ml/kg, b.w.). Positive controls included sitagliptin and vildagliptin. Values are mean ± SEM, $n = 4$ (*in vitro* DPP-IV enzymatic activity) and $n = 6$ (oral glucose tolerance test, plasma insulin level and *in vivo* DPP-IV enzymatic activity); *, **, ***$P < 0.05$–0.001 versus controls.

3B ($P < 0.05$–0.01) and enhanced plasma insulin levels at 30 min ($P < 0.01$) in STZ-induced type-2 diabetic rats (Figure 3C). EEHF (250 mg/5 ml/kg, b.w.) also decreased the plasma DPP-IV enzymatic activity at 30 and 180 min ($P < 0.05$), respectively. Sitagliptin and vildagliptin decreased DPP-IV enzyme level in a consistent manner ($P < 0.001$; Figure 3D).

EEHF and gut sucrose content

Oral gavage of EEHF (250 mg/5 ml/kg, b.w.) with sucrose (2.5 g/5 ml/kg) resulted in a significant amount of undigested sucrose at 30 and 60 min in the stomach and upper GIT, whereas at 60 and 120 min in the middle and lower GIT ($P < 0.05$–0.001; Figure 4A–D). In the caecum and large intestine of experimental animals, a very small concentration of undigested sucrose was found at 120 and 240 min ($P < 0.05$; Figure 4E and F).

EEHF and intestinal glucose absorption

The supplementation of glucose with EEHF (250 mg/5 ml/kg) resulted in a substantial decrease in GIT glucose absorption ($P < 0.05$–0.001; Figure 5A and B). EEHF (250 mg/5 ml/kg) demonstrated a significant amount of reduction in glucose absorption at 10, 15, 20 and 25 min ($P < 0.05$–0.001; Figure 5A), respectively. The area under the curve represented a 28% decline in glucose absorption ($P < 0.01$; Figure 5B).

EEHF and IDs enzyme and GI motility

Oral gavage of EEHF (250 mg/5 ml/kg) substantially suppressed IDs activity ($P < 0.05$; Figure 5C). EEHF at the same dose also augmented GI motility ($P < 0.05$; Figure 5D). Acarbose (200 mg/5 ml/kg) and bisacodyl (1 mg/5 ml/kg) inhibited disaccharidase activity by 53% and induced GI motility by 87% ($P < 0.001$; Figure 5C and D) respectively.

Structural identification of purified extract

The pharmacologically possible active molecules characterized from EEHF bark are listed in Table 1 (Figure 6). We collected six major peaks from the crude extract of *H. fomes* via RP-HPLC. Further investigation using LC-MS reported the presence of compounds of molecular masses 577.2, 289.1, 447.2 and 593.1 Da are corresponded with previously isolated phytochemicals procyanidins, epicatechin, quercitrin and proanthocyanidins, accordingly (Table 1).[7, 29–31]

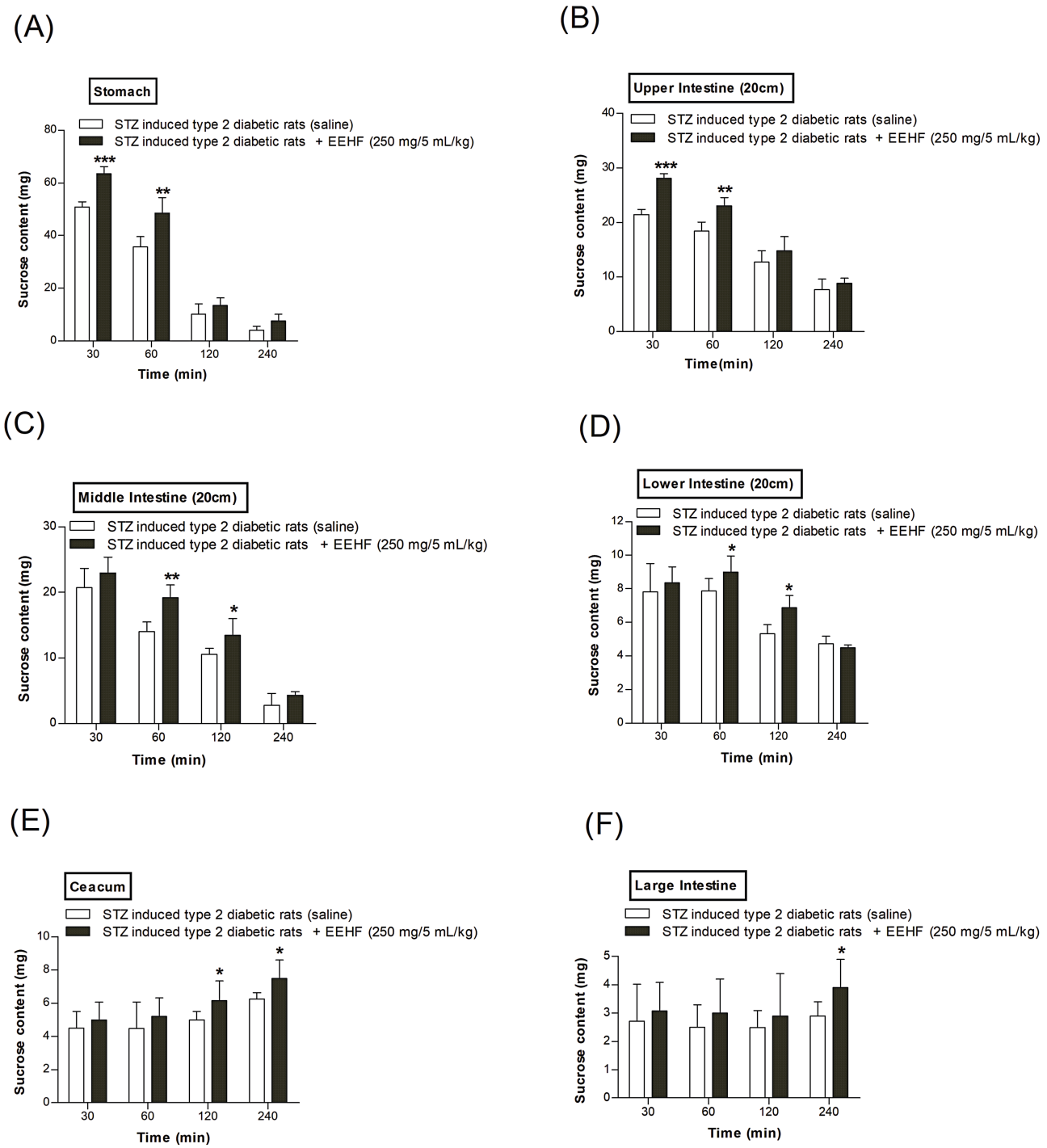

Figure 4 Effects of EEHF on (A–F) gut sucrose malabsorption in STZ-induced type-2 diabetic rats. Fasting (24 h) rats given a sucrose solution (2.5 g/5 ml/kg, b.w.) with or without EEHF (250 mg/5 ml/kg, b.w.). Values are mean ± SEM, $n = 6$; *· **$P < 0.05$–0.01 versus STZ-induced type-2 diabetic rats alone.

Discussion

Heritiera fomes bark is traditionally used for diabetes.[9] Previous studies have revealed that it had beneficial effects in treating T2DM[10] and that its bark and leaves had antidiabetic activity.[11, 12] In this study, we intended to investigate the *in vitro* and *in vivo* antihyperglycemic activity of ethanol extract of *H. fomes* (EEHF) bark to further understand the mechanisms contributing to such activity[7] and provide additional scientific evidence to support the ethnomedicinal use of this plant as an antidiabetic agent.

In clonal pancreatic β-cells and isolated islets, EEHF induced insulin secretion in a dose-dependent manner. Non-toxic concentrations of EEHF also enhanced insulin secretion induced by glucose with/without insulin modulators. Tolbutamide (first-generation sulfonylurea and K_{ATP}-channel blocker drug) or KCl (depolarizing agent) was used to investigate the potential involvement of single or multiple pathways like adenylate cyclase/cAMP or the phosphatidylinositol system in the insulin-releasing effect of EEHF. In the presence of the insulin-release inhibitors such as diazoxide and verapamil,

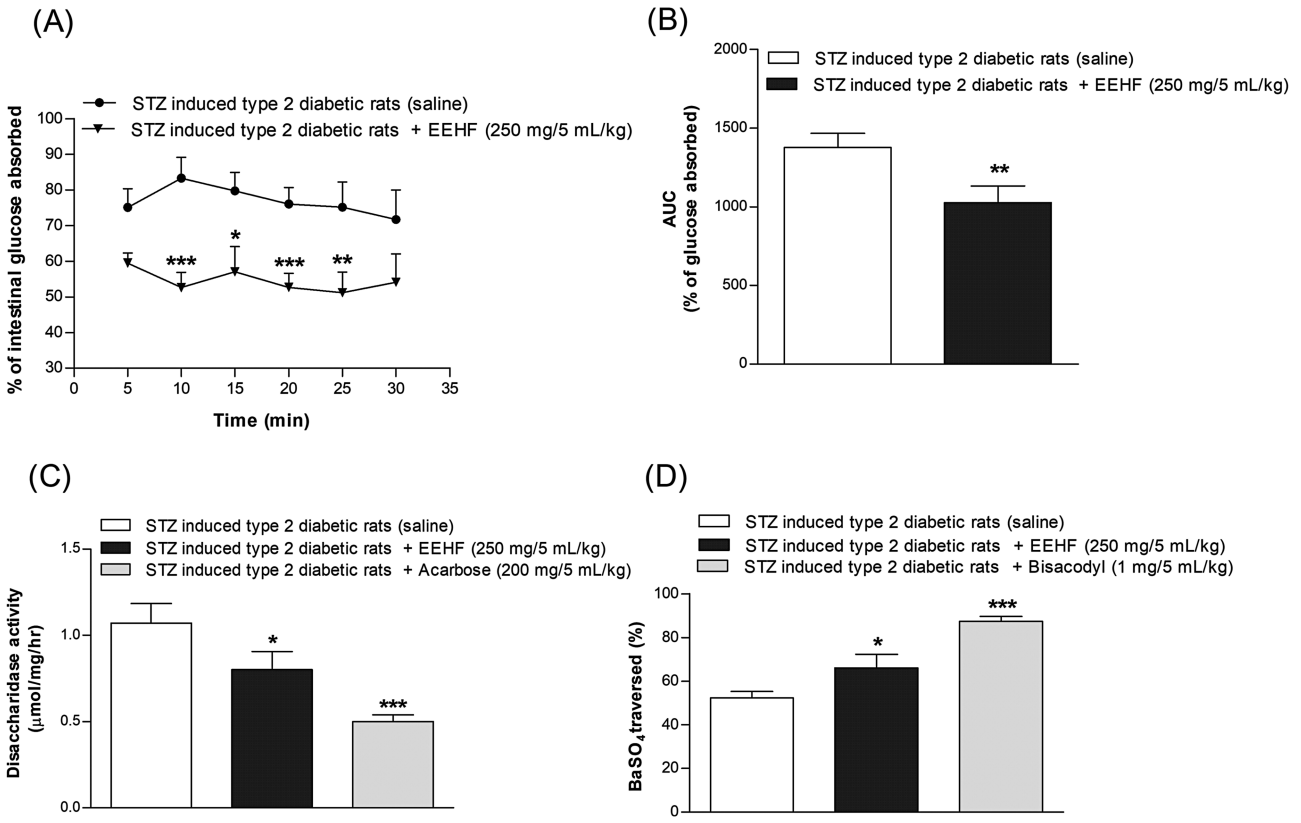

Figure 5 Effects of EEHF on (A & B) gut perfusion, (C) IDs enzymatic activity and (D) GI motility in STZ-induced type-2 diabetic rats. After a 36-h fast, intestinal perfusion was performed by glucose (54 g/l) with/without EEHF (250 mg/5 ml/kg, b.w.). $BaSO_4$ was administered 1 h following the initial oral dosing. Positive controls for IDs enzymatic activity and GI motility tests were acarbose (200 mg/5 ml/kg, b.w.) and bisacodyl (1 mg/5 ml/kg, b.w.), respectively. Values are mean ± SEM, $n = 6$; *, **, ***$P < 0.05$–0.001 versus STZ-induced type-2 diabetic rats alone.

Table 1 Peak samples of ethanol extract of *H. fomes* bark obtained from preparative RP-HPLC and their predicted identities using LC-MS

Peak samples	Retention time (min)	Theoretical molecular Wt. (Da)	Found molecular weight (Da)	Predicted compounds
P_1	11.07	578.5	577.2	Procyanidins
P_2	12.08	290.3	289.1	Epicatechin
P_3	13.50	–	561.2	Unknown
P_4	17.94	–	449.1	Unknown
P_5	20.02	448.03	447.2	Quercitrin
P_6	23.09	592.5	593.1	Proanthocyanidins

Peaks were separated using a Kinetex 5m F5 LC column (150 × 4.6 mm) on a Spectra System LC (Phenomenex). The mass-to-charge ratio (*m/z*) was measured against peak intensity.

the insulin-releasing effects of EEHF were reduced. This suggests that EEHF acts via closure of the K_{ATP}- channels and depends on Ca^{2+}- channels to induce insulin release. These findings were confirmed further by the effects of EEHF on depolarization of membrane and $(Ca^{2+})_i$ in BRIN-BD11 cells. The insulin-releasing effect of EEHF was further improved in the presence of IBMX, a well-established inhibitor of cAMP phosphodiesterase.[32] Interestingly, it has been previously hypothesized that the cAMP pathway may contribute to the antioxidant and anti-inflammatory activity of EEHF.[11]

Any signal transduction impairment in muscle or reduced glucose transporter type 4 (GLUT4) translocation can trigger insulin resistance; therefore, medicines or adjunct treatments that are able to increase glucose uptake are ideal antidiabetic agents.[33] Results showed that EEHF enhanced glucose uptake in 3T3L1 cells, and this is predicted to be facilitated through the adenosine monophosphate-activated protein kinase (AMPK) pathway.[34] This result was observed both with and without insulin. A previous report indicated that a flavanone called naringenin could inhibit the insulin-induced GLUT4 translocation regulator, phosphoinositide 3-kinase (PI3K), resulting in increased insulin-stimulated glucose uptake in 3T3L1 adipocytes.[35] As flavonoids are known to be present in *H. fomes*, it could be hypothesized that such phytoconstituents are responsible for the increase in glucose uptake observed for EEHF.[36]

When proteins, such as insulin, are glycated, this decreases their pharmacological activity.[37, 38] Protein glycosylation plays a key role in the complication of DM.[39] As EEHF substantially reduced protein glycation, it may protect from long-term complications associated with diabetes by inhibiting the glycation of insulin. This may be linked with the presence of antioxidant phytoconstituents such as flavonoids.[40, 41]

EEHF reduced starch digestion by delaying carbohydrate digestion and glucose absorption into the bloodstream. A previous study has reported that phytoconstituents such as alkaloids and flavonoids present in *H. fomes* had α-glucosidase inhibitory activity.[42] In addition to that, the bark of *H. fomes* consists of a large proportion of fibres, which may also interfere with nutritional absorption.[43] Previous studies

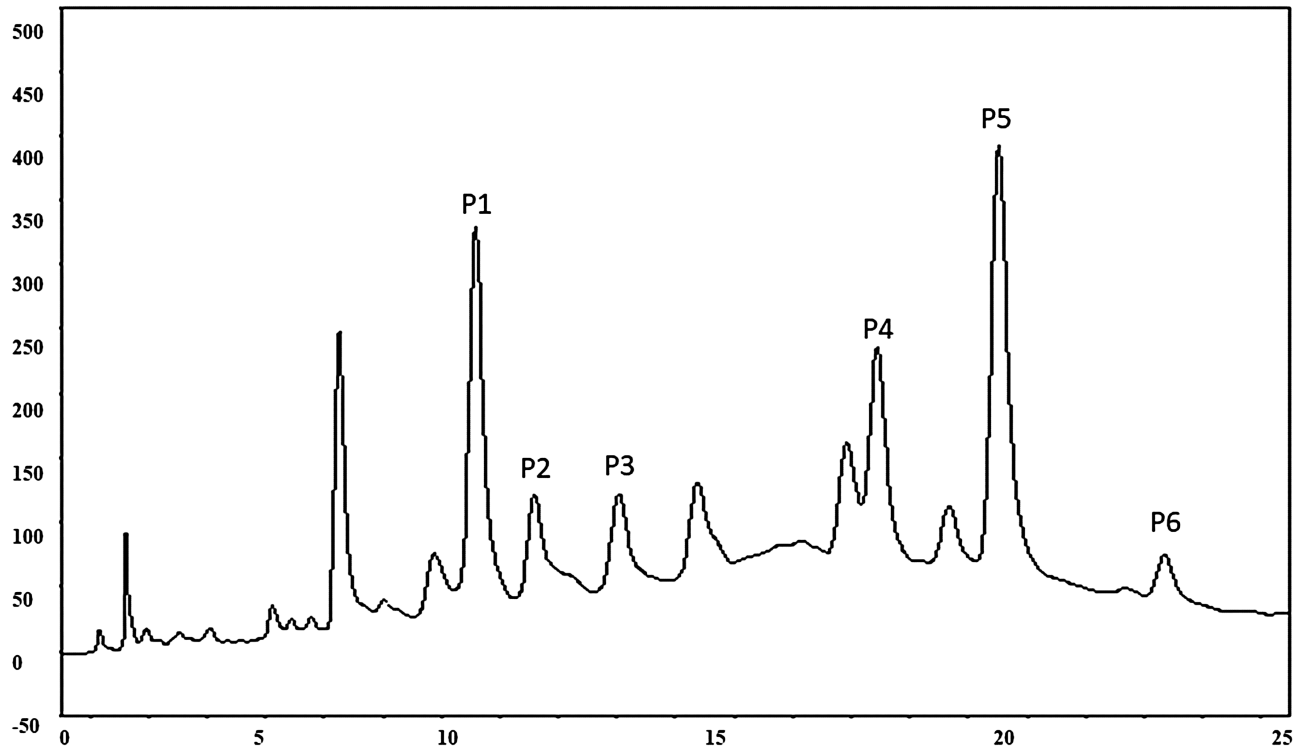

Figure 6 RP-HPLC chromatogram of ethanol extract of *Heritiera fomes*. At a flow rate of 1.0 ml/min, the column was equilibrated with 0.12 percent (v/v) TFA/water. The eluting solvent was maintained at a gradient ratio (acetonitrile concentration increased from 20% to 70% within the eluting solvent) for 40 min cycle. At 214 and 256 nm, UV detection was set to each run with a 1 mg/ml sample injected. The chromatogram provides the details on the peaks associated with *H. fomes* bark, which are denoted by numbers (P_1 to P_6).

have shown that many antidiabetic plants are able to lower gastrointestinal glucose absorption and diffusion.[44] A dialysis model used to evaluate glucose diffusion was a proxy to accurately imitate gastrointestinal absorption.[23] Glucose passage across the dialysis membrane was slowed down by different concentrations of EEHF. A known GIT inhibitor, guar, dramatically reduced glucose diffusion. The lowering in glucose diffusion and absorption observed for EEHF may contribute to glucose homeostasis.

GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are two endogenous incretin hormones with insulin-releasing and blood glucose-lowering effects via reducing glucagon secretion in the intestine.[45] DPP-IV enzymatic cleavage of GLP-1 and GIP affords two inactive peptides GLP-1 (9-36) and GIP (3-42), respectively.[46] Therefore, DPP-IV enzyme antagonists can be utilized to treat diabetes as they improve GLP-1 and GIP half-life.[47] Our results showed that EEHF decreased DPP-IV *in vitro* enzymatic activity in a dose-dependent manner, and this effect was also observed *in vivo* at different time points. Phytoconstituents such as flavonoids, previously reported in *H. fomes* and which have been reported to act as DPP-IV inhibitors, may contribute towards the observed effects.[48]

In T2DM rats, EEHF therapy improved acute glucose tolerance. It inhibited glucose absorption *in situ* with significant quantities of non-absorbed sucrose remaining in the GIT. *In vitro* studies revealed that EEHF restricted both the digestion of carbohydrates and the absorption of glucose from the GIT. EEHF decreased IDs activity and enhanced intestinal motility in a $BaSO_4$ milk study. Hence, it reduces the amount of time available for carbohydrates digestion and absorption[25] and helped in improving post-prandial hyperglycaemia regulation. These effects, in agreement with previous findings, are indicating that *H. fomes* consist of high fibre and that forming carbohydrate complex upon ingestion and resisting post-prandial carbohydrate absorption.[7] It should be noted that dietary fibre has numerous impacts on the GIT, including alterations in content viscosity and transit time. These effects also impact food movements across the GIT, enzymatic hydrolysis of nutrients and nutrient interaction with the absorbing mucus layer.[49] This reflects the rapid sucrose hydrolysis and absorption in the upper GIT.

Finally, a series of chromatographic and mass spectrometric techniques was used to identify and characterize active compound/s. Based on our primary screening, we have found several interesting peak fractions, which have been suggested to be active molecules involved in EEHF-mediated antihyperglycemic action. Furthermore, the molecular masses of peak fractions P_1, P_2, P_5 and P_6 are associated with procyanidins, epicatechin, quercitrin and proanthocyanidins.[7,29–31] Further study is recommended to illustrate and re-confirm currently obtained phytoconstituents by Nuclear Magnetic Resonance (NMR). The previous studies reported that *H. fomes* contains procyanidins and proanthocyanidins,[7] which facilitate insulin secretion from pancreatic β-cells.[50–56] Procyanidins also promote GLUT4 translocation in the muscle tissue via phosphorylation of AMPK and downstream signalling in ICR mice.[53] Additionally, epicatechin has antioxidant and insulinogenic activity, and it also has insulin-like properties.[50,54] Therefore, the existence of these phytomolecules may contribute to the antidiabetic action of EEHF.

Conclusion

The present findings showed that EEHF bark stimulates the insulin release from pancreatic β-cells and primary islets. It can control hyperglycaemia via several mechanisms to improve glucose homeostasis, including delaying the absorption of glucose and blocking the effect of the DPP-IV enzyme. Procyanidins, epicatechin and proanthocyanidins are some of the phytoconstituents that are predicted to play a vital role in insulin secretion and action. This indicates that EEHF may be beneficial as a nutritional adjunct therapy in the management of T2DM. This warrants further research to explore the effectiveness of this plant and its phytomolecules as an antidiabetic agent in humans.

Supplementary Material

Supplementary data are available at *Journal of Pharmacy and Pharmacology* online.

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Author Contributions

Y.H.A.A.-W. and P.A. conceptualized and supervised the project; P.A. carried out the study and interpreted the results; P.A. and S.A. drafted the initial version of the manuscript; P.A. and V.S. modified the revised manuscript, and all contributors indorsed the revised edition.

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Conflict of Interest

With regard to these findings, none of the authors have a conflict of interest.

Data Availability Statement

The data underlying this article cannot be shared publicly due to ethical/intellectual property. However, the data will be shared on reasonable request to the corresponding author.

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