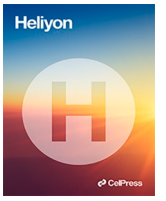
Heliyon 10 (2024) e38703

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Antihyperglycemic activity of a novel polyherbal formula (HF344), a mixture of fifteen herb extracts, for the management of type 2 diabetes: Evidence from *in vitro, ex vivo,* and *in vivo* studies

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A R T I C L E I N F O

Keywords:
Polyherbal formula
Type 2 diabetes mellitus
Antihyperglycemic
Glucose transporters
Oral glucose tolerance test
Piperine

A B S T R A C T

Antihyperglycemic effects of a novel polyherbal formula (HF344), comprising fifteen Thai herbal extracts, were elucidated for pharmacological mechanisms and potential for managing type 2 diabetes mellitus, by employing *in vitro*, *ex vivo*, and *in vivo* approaches. LC/MS analysis of HF344 extract revealed several phytoconstituents, with piperine identified as the major active compound. HF344 extract significantly enhanced insulin secretion in RINm5F cells *in vitro* and inhibited glucose uptake into the everted sacs of the mouse small intestine *ex vivo* in a concentration-dependent manner compared to the control ($p < 0.05$). It exhibited potent α-glucosidase inhibition *in vitro*, with an IC50 of 96.74 μg/mL. Moreover, HF344 extract upregulated mRNA levels of GLUT1 in L6 skeletal myoblasts, suggesting increased glucose uptake into skeletal muscle. In addition, *in vivo* antihyperglycemic effects were assessed in streptozotocin (STZ)-nicotinamide (NA)-induced diabetic mice. Acute oral toxicity testing confirmed the HF344 extract's safety, with an LD50 exceeding 2000 mg/kg. Oral administration of HF344 extract (500 and 1000 mg/kg) in STZ-NA-induced diabetic mice significantly reduced the area under the fasting blood glucose (FBG)-time curve (AUC) in the oral glucose tolerance test (OGTT) model and treatment for 28-day reduced the FBG levels as compared with control ($p < 0.05$). This was accompanied by increased serum insulin levels and improved insulin resistance. HF344 extract also demonstrated a concentration-dependent inhibitory effect on malondialdehyde (MDA) production *in vitro*, with an IC50 of 7.24 μg/mL. Oral treatment with HF344 extract decreased MDA production in the homogenized muscle *ex vivo* collected from STZ-NA-induced mice.

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https://doi.org/10.1016/j.heliyon.2024.e38703
Received 17 May 2024; Received in revised form 3 September 2024; Accepted 27 September 2024
Available online 1 October 2024

Furthermore, pretreatment with HF344 extract effectively restored the survival of RINm5F cells from STZ-induced damage. These findings suggest that HF344 is a promising polyherbal formula for managing blood glucose levels, enhancing insulin production, and providing antioxidant benefits in T2DM. Further research is required to evaluate the clinical efficacy and safety profiles of HF344.

1. Introduction

Type 2 diabetes mellitus (T2DM) remains a significant global health challenge, characterized by hyperglycemia resulting from insulin resistance and impaired insulin secretion. The World Health Organization (WHO) reported that the global number of people with diabetes increased from 108 million in 1980 to 422 million in 2014. Additionally, diabetes and diabetes-related kidney disease were responsible for approximately 2 million deaths in 2019, underscoring the significant global health burden posed by this disease [1]. According to 2021 data from the International Diabetes Federation (IDF), the global prevalence of diabetes was 9.8 %, with 44.7 % of cases undiagnosed. Additionally, 32.6 % of diabetes-related deaths occurred in people under 60 years old, and the diabetes-related health expenditure per person was 1838.4 USD [2]. Currently, around 90 % of diabetes cases in Thailand are classified as type 2 diabetes mellitus (T2DM), which is a leading cause of mortality in the country. Diabetes mellitus contributes to approximately 15 % of the mortality rate among chronic non-communicable diseases in Thailand [3]. Although there are many effective modern medications for managing diabetes, they often have undesirable side effects, leading many patients to seek alternative treatments like herbal remedies, either as sole therapies or in conjunction with conventional medications [3].

In general, herbal medicines derived from natural plants confer moderate therapeutic benefits with fewer adverse reactions than conventional drugs, but there is limited scientific evidence to support their efficacy on a biochemical and pharmacological basis. While certain herbs are believed to have the potential to lower blood glucose levels or alleviate diabetic symptoms, the precise mechanisms of action, particularly in polyherb formulations, are not well understood [4].

Diabetes, oxidative stress, and inflammation are closely interconnected. Under normal blood sugar levels (euglycemia), mitochondria efficiently produce energy and manage reactive oxygen species (ROS) using natural antioxidant enzymes. However, in

Table 1
A novel set of Thai traditional herbal plants (HF344), unified from three sets of herbs categorized into antidiabetic, antioxidative, and anti-inflammatory effects, respectively.

No	Scientific name	Categorical pharmacological effects					Ref.
		Anti-diabetic			Anti-oxidative	Anti-inflammatory	
		Inhibition on alpha-glucosidase	Stimulation insulin production	Other mechanisms[a]		Reduce inflammatory mediators	
1	*Allium sativum* L.	✓	✓	✓	↓MDA, ↑ GSH, SOD, CAT	↓TNF-α, IL-1β, iNOS	[6–8]
2	*Suregada multiflora* (A. Juss.) Baill.	✓					[9]
3	*Acanthus ebracteatus* Vahl	✓			✓	↓MMP-9	[10–12]
4	*Piper nigrum* L.	✓			✓	✓	[13–15]
5	*Cleome viscosa* L.					✓	[16]
6	*Bridelia ovata* Decne.				✓	✓	[17]
7	*Cyperus rotundus* L.	✓			✓	✓	[18,19]
8	*Terminalia chebula* Retz.	✓	✓	✓	↑ GSH, CAT	✓	[20,21]
9	*Alpinia galanga* (L.) Willd.			✓			[22]
10	*Curcuma longa* L.	✓			↑ GSH, SOD, CAT	✓	[23–25]
11	*Curcuma zedoaria* (Christm.) Roscoe	✓				✓	[26,27]
12	*Senna alexandrina* (Christm.) Roscoe var. alexandrina	✓			↓lipid peroxidation, ↑ GSH, SOD, CAT, Nrf2	↓ fatty liver inflammation and fibrosis, ↓NO, PGE2, IL-1β	[28,29]
13	*Smilax glabra* Roxb.	✓	✓				[30]
14	*Smilax corbularia* Kunth.					↓PGE2, TNF-α, NO	[31,32]
15	*Terminalia arjuna* Wight & Arn.		✓	✓	↓lipid peroxidation, ↑GSH, CAT		[33]

Abbreviations: CAT: catalase; FBG: fasting blood glucose; GSH: Glutathione; IL-1β: interleukin-1 beta; MDA: malonaldehyde; MMP-9: matrixmetalloproteinase-9; NO: Nitric Oxide; NOS: Nitric Oxide Synthase; Nrf2: nuclear factor erythroid 2-related factor 2; PGE2: Prostaglandin E2; ROS: reactive oxygen species; SOD: superoxide dismutase; TNF-α: tumor necrosis factor-alpha.

[a] Others mechanisms such as improvement of glycosylated hemoglobin, restoring the function of islet β cells, stimulating GLUT4 expression, decrease Homeostatic Model Assessment for Insulin Resistance (HOMR-IR), increase SIRT1 (Sirtuin 1).

hyperglycemia, excess glucose enhances oxidative phosphorylation, leading to the overproduction of ROS, which disrupts mitochondrial function and causes oxidative stress. This results in damaged pancreatic β-cells, impairing insulin secretion and causing endoplasmic reticulum stress. In peripheral tissues, excessive ROS interferes with insulin signaling pathways, contributing to insulin resistance. Chronic hyperglycemia also triggers local and systemic inflammation through the release of cytokines and chemokines. Consequently, the antioxidant and anti-inflammatory properties of certain herb extracts can enhance their antihyperglycemic effects by targeting these multiple pathways, improving the pathological conditions associated with diabetes [5].

In this study, a novel polyherbal formulation nominated as HF344 was developed, which is a powdered mixture of fifteen traditional Thai herbs thought to be beneficial for reducing diabetes-related complications and symptoms. The objective of this research was to explore the pharmacological properties of HF344 and identify its mechanism of action, particularly for its antihyperglycemic effects. To achieve this, *in vitro, ex vivo,* and *in vivo* studies were conducted to elucidate the effects of HF344 on glucose metabolism and other diabetes-related parameters. Additionally, we performed an acute oral toxicity test to assess the safety of this polyherbal formulation.

2. Methods

2.1. Designing of a novel polyherbal formulation

In the present study, we built three sets of Thai traditional herbs that may exhibit antidiabetic, antioxidative, and anti-inflammatory effects, respectively, and then unified these three sets as a novel polyherbal recipe, which was nominated as HF344. The unified set of herbs consists of fifteen herbs and are listed in Table 1.

To formulate a novel herbal preparation, HF344, containing the above fifteen herbal components, all the plants listed in Table 2 were purchased and identified as authentic botanicals by a professional botanist and then deposited at the Sireeruckhachati Herbarium, Mahidol University, Bangkok, Thailand.

A crude mixture of fifteen plant materials (listed in Table 2) underwent initial washing, cleaning, and drying at 55 °C for 6 h. Subsequently, the dried herbs were combined and ground into a powder. The resulting powdered mixture was then filtered through gauze. Additionally, 2 % magnesium sulfate ($MgSO_4$ or Epsom salt) was incorporated into the formula. The polyherbal powder was extracted using the maceration method, where 160 g of the herbal mixture was immersed in 300 ml of 95 % ethanol for 3 days, with the process repeated 3 times. The supernatant was collected, and the ethanol was evaporated. After drying, the weight of the ethanol extract was found to be 23.22 g, representing a yield of 14.51 %. The obtained ethanolic extract powder was stored in a closed container at 4 °C until use.

Table 2
Fifteen herbal components incorporated in the novel polyherbal formula, HF344.

No	Scientific name	Family name	Common name	Vernacular name	Voucher/ collector's No.	Part used	% w/ w
1	*Allium sativum* L.	Amaryllidaceae	Garlic	kra thiam, kra thiam chin, kra thiam khao	PBM-006314	Rhizome	5
2	*Suregada multiflora* (A. Juss.) Baill.	Euphorbiaceae	False lime	khan thong phayabat, khan thong, duk mai	PBM-006316	Wood	2
3	*Acanthus ebracteatus* Vahl	Acanthaceae	Holly-leaved mangrove, Sea holly	(ngueak pla mo, nang kreng, ngueak pla mo nam ngoen	PBM-006318	Whole Plant	3
4	*Piper nigrum* L.	Piperaceae	Pepper	phrik thai, phrik noi	PBM-006320	Fruit	41
5	*Cleome viscosa* L.	Cleomaceae	–	phak sian phi, phak som sian phi	PBM-006322	Whole Plant	5
6	*Bridelia ovata* Decne.	Phyllanthaceae	–	ma ka, kong keep, mat ka, sam sa	PBM-006324	Leaves	5
7	*Cyperus rotundus* L.	Cyperaceae	Nut grass	ya haeo mu, ya khon mu	PBM-006328	Rhizome	5
8	*Terminalia chebula* Retz.	Combretaceae	Myrabolan wood	samo thai, samo ap phaya, ma na	PBM-006329	Fruit	5
9	*Alpinia galanga* (L.) Willd.	Zingiberaceae	Galangal, Greater galangal	Kha, kha yuak, kha luang, sa-e-choei	PBM-006331	Rhizome	5
10	*Curcuma longa* L.	Zingiberaceae	Turmeric	Khamin, khamin kaeng, khamin chan	PBM-006332	Rhizome	3
11	*Curcuma zedoaria* (Christm.) Roscoe	Zingiberaceae	Rose turmeric	khamin oi, khamin khuen, la-miat	PBM-006333	Rhizome	5
12	*Senna alexandrina* (Christm.) Roscoe var. alexandrina	Fabaceae	Alexandrian Senna, East Indian Senna, Tinnevelly Senna	ma kham khaek	PBM-006334	Leaves	5
13	*Smilax glabra* Roxb.	Smilacaceae	China root, Sarsaparilla	ya hua, khao yen tai	PBM-006335	Rhizome	2
14	*Smilax corbularia* Kunth.	Smilacaceae	China root, Sarsaparilla	khao yen nuea, hua khao yen nuea	PBM-006336	Rhizome	2
15	*Terminalia arjuna* Wight & Arn.	Combretaceae	–	samo that	PBM-006330	Fruit	5

2.2. Quantitative phytochemical analysis

Quantitative analysis of herbal components using ultrahigh-performance liquid chromatography with diode-array detector quadrupole Orbitrap mass spectrometry (UHPLC-DAD-Q-Orbitrap-MS) was performed using a Vanquish UHPLC system (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a Thermo Scientific Vanquish Binary Pump F, Thermo Scientific Vanquish Split Sampler FT, Thermo Scientific Vanquish Column Compartment H, and Thermo Scientific Vanquish Diode Array Detector FG, coupled with a Thermo Scientific Orbitrap Exploris™ 120 mass spectrometer. The separation was conducted on a BDS Hypersil C18 column (50 × 2.1 mm i.d., 2.4 μm). The mobile phases were (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in methanol. A mobile phase gradient program was set up with 100 % A for 2 min, followed by a linear increase from 0 % to 100 % B in A over 8 min, and then held at 100 % B for 2 min. Before each injection, the column was equilibrated with 100 % A for 2 min. The column temperature was maintained at 25 °C with a constant flow rate of 1.0 mL/min. DAD detection was set at a wavelength of 254 nm. The injection volume was set to 2 μL for all samples. Mass spectrometric analysis was conducted in both positive and negative modes using internal mass calibration EASY-IC™. The ion source type was Heated-ESI. Spray voltage settings were static, with positive ion set to 3500 V and negative ion set to 2500 V. Nitrogen gas mode was set to static with the following flow settings: sheath gas at 60 Arb, Aux gas at 15 Arb, and sweep gas at 2 Arb. The ion transfer tube temperature was maintained at 350 °C, while the vaporizer temperature was also set to 350 °C. Full scan mode range was set from 200 to 1000 *m/z* with a resolution of 60,000 and RF Len at 70 %. ddMS^2 mode was triggered with an intensity threshold of 5.0 x 10^5. The MS^2 parameters included an isolation window of 1.5 *m/z*, collision energy type set to normalized, orbitrap resolution set at 15,000, and scan range mode set to automatic. The instrument was controlled and analyzed using Chromeleon™ Chromatography Data System software (Thermo Fisher Scientific). Recorded chromatograms were visualized and analyzed using FreeStyle software (Thermo Fisher Scientific). Compounds were identified or tentatively identified based on their mass spectral data.

The piperine content was determined by high-performance liquid chromatographic (HPLC) analysis following the method described in a previous study [34]. The HPLC system used was the Prominence LC-20AD (Shimadzu, Kyoto, Japan), which was equipped with a degasser (DGU-20A), an autosampler (SIL-10AF), a UV–VIS detector (SPD-20A), and a column oven (CTO-20A), all equipped with LC Solution software. Chromatographic separation was carried out using a BDS Hypersil C18 column (150 × 4.6 mm, i.d. 5 μm) with a guard column (4 × 10 mm, i.d. 5 μm, Thermo Fisher Scientific). The mobile phase consisted of acetonitrile: 1 % acetic acid in water (48:52, v/v) with a flow rate of 1.0 mL/min. The total run time was 20 min. The injection volume was 10 μL. The UV detector and oven temperature were set at 341 nm and 40 °C, respectively. Standard piperine and samples were accurately weighed and dissolved in 50 % acetonitrile, then filtered through a 0.45 μm nylon membrane filter before being analyzed by HPLC in triplicate.

2.3. Antihyperglycemic activities of HF344 extract in vitro and ex vivo

2.3.1. Insulin secretion stimulatory assay in vitro

The rat insulinoma cell line RINm5F (CRL-2058™, passage number 19–24), obtained from American Type Culture Collection (ATCC®) was seeded at a density of 1x10^5 cells/mL. They were cultured in a 96-well microplate with RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 units/mL penicillin. The cells were maintained in a humidified incubator at 37 °C with 95 % air/5 % CO_2 for 24 h. Subsequently, the cells were incubated with various concentrations of HF344 extract (1–1000 μg/mL, dissolved in DMSO) or with glibenclamide (10 μg/mL) as the positive control for an additional 24 h. After the incubation, 5 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) solution in phosphate-buffered saline (PBS) was added to each well, and the microplate was incubated for another 4 h. Following this, the medium was carefully aspirated, and 100 μL of DMSO was added to each well to dissolve the formed formazan crystals. The absorbance of the formazan solution, which indicates cell viability, was measured at wavelengths of 550 nm and 595 nm using a microplate spectrophotometer [35,36]. The percentage of cell viability was calculated using the following equation (1):

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of test} - \text{Absorbance control}}{\text{Absorbance of control}} \times 100 \quad \text{(Eq. 1)}$$

To examine the effect of HF344 extract on insulin secretion from RINm5F cells, these cells were cultured in 24-well plates at 70–90 % confluency (1x10^6 cells/well) at 37 °C with 5 % CO_2 for 72 h. Subsequently, the cells were washed three times with Krebs-Ringer bicarbonate (KRB) buffer and pre-incubated with KRB buffer containing 1.1 mM glucose for 40 min at 37 °C. The buffer was then replaced with KRB containing HF344 dissolved in DMSO at concentrations of 1, 10, and 100 μg/mL or glibenclamide (10 μg/mL) as a positive control. The cells were incubated at 37 °C for 6 h, allowing the test compounds to affect insulin secretion [35]. Afterward, the supernatant was collected for insulin secretion determination using the Insulin High Range Assay kit HTRF® (Cisbio).

2.3.2. Glucose uptake into everted sacs of the small intestine ex vivo

The method to assess the effect of HF344 extract on glucose uptake in everted sacs of the mouse small intestine *ex vivo* was adapted from a previous study [37] with some modifications. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmacy, Mahidol University, Thailand, under permission approval number PYR 003/2019. The experiments were conducted in accordance with ethical principles and guidelines for the use of animals by the National Research Council of Thailand. After 7 days of adaption period, male ICR mice (35–40 g), obtained from the National Laboratory Animal Center, Mahidol University (Bangkok, Thailand), were fasted for 3–4 h, followed by euthanasia by CO_2 overdose. Then, the jejunum was promptly removed, washed with PBS, and everted after ligating one end. Both ends of the jejunum were tied to form a sac, into which 0.5 mL of

Krebs-Hanseleit solution was introduced and maintained at 37 °C. Then, the everted sac was then placed into a volumetric flask containing glucose (140 mg/dL) along with either the HF344 extract (1.25–10 mg/mL) or sodium fluoride (NaF) at a concentration of 2 mM as a positive control. This setup was kept in a shaking water bath at 37 °C, with a gas mixture of 95 % O2/5 % CO_2 supplied via a tube for 30 min. At the end of the experiment, the serosal fluid (inside the sac) was collected and analyzed for glucose concentration using a Glucose Assay Kit (Abcam®). The obtained glucose concentrations were compared between the test and control groups.

2.3.3. mRNA expressions of GLUT1 and GLUT4 in myoblasts in vitro

The H9c2 rat cardiomyoblasts and L6 skeletal myoblast were acquired from the ATCC, CRL-1446, and ATCC, CRL1458™, respectively. They were cultured in 96-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS and 0.1 % Penicillin-Streptomycin (P/S: 10,000 U/mL) at 37 °C and 5 % CO_2 for 24 h in an incubator (Thermo Scientific, USA). The cell culture medium was aspirated and changed, and HF344 extract at concentrations ranging from 31.25 to 1000 μg/mL was incubated with the cells. Cell viability, determined by MTT assay, was assessed in the same manner as described earlier for the cell line, RINm5F.

The mRNA expression levels of GLUT1 and GLUT4 were analyzed using a real-time PCR [38]. H9c2 and L6 cells were cultured in DMEM supplemented with 10 % FBS and P/S solution. The cells were then transferred to FBS-free DMEM and incubated for 24 h before being stimulated with HF344 for another 24 h. Subsequently, the cells were washed with PBS, and total mRNA was extracted using an mRNA purification kit (Qiagen-RNeasy). The obtained mRNA was analyzed for the mRNA contents of GLUT1 and GLUT4 using quantitative reverse transcription PCR (qRT-PCR), employing the KAPA SYBR® FAST one-step quantitative RT-PCR kit (KAPA Biosystems, Canada).

The primers of rat GLUTs were designed and ordered as follows: GLUT1 (sense, 5′- CATCGTCGTTGGGATCCTTA-3'; antisense, 5′-GAGACAGTAGAGGCCACAAGTCT-3′), GLUT4 (sense, 5′- CCCCCGATACCTCTACAT-3'; antisense, 5′- GCATCAGACACATCAGCCCAG -3′), and GAPDH (sense, 5′- CAGTCAAGGCTGAGAATGG-3'; antisense, 5′-CGACATACT CAGCACCAGC-3′). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization, and expressed as fold change according to the comparative cycle threshold (CT) analysis.

2.3.4. α-Glucosidase activity in vitro

The α-glucosidase inhibitory activity of HF344 was evaluated using a method described previously [39], with some modifications. Briefly, 10 μL of either the HF344 extract or acarbose (a positive control) was gently mixed with 40 μL of α-glucosidase enzyme (0.1 U/mL) in 0.1 M phosphate buffer (pH 6.9). After pre-incubation at 37 °C for 10 min, the reaction was initiated by adding 50 μL of 2 mM p-nitrophenyl α-D-glucopyranoside (pNPG), and the solution was further incubated at 37 °C for 20 min. The reaction was stopped by adding 100 μL of 1 M Na_2CO_3, and the released *p*-nitrophenol was measured in triplicate at 405 nm using a microplate reader (Tcan, Switzerland). The inhibitory percentage was calculated, and the IC50 was determined from the HF344 concentration and the corresponding inhibition percentage.

2.3.5. Antihyperglycemic activity of HF344 extract in vivo

2.3.5.1. Animals. Male ICR mice (30–40g) were housed in the Animal Center at the Faculty of Pharmacy, Mahidol University, under constant conditions of 23 ± 2 °C and a 12-h light-dark cycle. They had free access to standard food and water ad libitum. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmacy, Mahidol University (Approval number: PYR003/2023).

2.3.5.2. Acute oral toxicity test. The acute oral toxicity of HF344 extract was evaluated according to the Organization for Economic Cooperation and Development (OECD) test guideline 425, using a limit test approach [40]. Five nulliparous, non-pregnant female mice weighing between 30 and 35 g were used per group for this experiment. Prior to the test, the mice underwent a 7-day adaptation period, after they were fasted for 3–4 h before administration of the test substance. For the treatment group, HF344 extract was prepared at a concentration of 2000 mg/kg, suppended in 1 % carboxymethyl cellulose sodium (CMC-Na), and given via oral gavage at a dose volume of 1 mL/100g. The control group received only the 1 % CMC-Na solution. The first mouse in the treatment group was monitored closely for 30 min after dosing to observe any signs of acute toxicity. If the first mouse survived without showing signs of toxicity, an additional 4 mice were dosed under the same conditions, resulting in a total of five mice in the treatment group. During the 14-day observation period, both groups were monitored closely for symptoms of toxicity, including changes in fur and skin color, eye appearance, respiration, convulsions, and diarrhea. This observation was particularly intensive during the first 4 h following dosing, with regular checks continuing throughout the study duration. Additionally, the body weights of the mice were recorded daily. At the end of the study, if three or more mice survived, the LD50 was concluded to be above the test dose of 2000 mg/kg, indicating a low level of acute toxicity. However, if three or more mice died, the limit test would be terminated, and further testing would be required to determine the actual LD50 value.

2.3.5.3. Induction of diabetes in mice. Diabetes was induced in mice, as described previously [41]. The procedure involved 12-h fasting of the mice, followed by intraperitoneal (i.p.) administration of 120 mg/kg nicotinamide (NA), dissolved in normal saline. Fifteen minutes later, the mice received a single i.p. injection of 180 mg/kg streptozotocin (STZ), freshly prepared in 0.1 M citrate buffer with a pH of 4.5. To mitigate the initial risk of STZ-induced hypoglycemia, a 10 % glucose solution was provided to all mice for the first 12 h after injection. Between 21 and 28 days post-induction, fasting blood glucose (FBG) levels were measured to confirm the induction of

diabetes. Blood samples were collected from the tail vein and analyzed immediately using a glucose meter (Accu-Chek®, Roche, Thailand). Mice with FBG levels exceeding 200 mg/dL were considered diabetic and employed in the subsequent experiments.

2.3.5.4. Oral glucose tolerance tests (OGTT). OGTT studies were conducted to investigate the hypoglycemic effect of HF344 extract on both normal and STZ-NA-induced diabetic (DM) mice, as reported previously [41]. In the study with normal mice, the animals were divided into three treatment groups. Group 1, the control group, received 1 % CMC-Na. Groups 2–4 were given HF344 extract at oral doses of 250, 500, and 1000 mg/kg, respectively, suspended in 1 % CMC-Na. All groups were fasted overnight for 12 h before the experiment. Mice were orally administered with 2 g/kg glucose and, after 30 min, the assigned treatments were given.

For an OGTT study in STZ-NA-induced DM mice, the mice were divided into six groups. Group 1, normal control mice, received 1 % CMC-Na. Group 2 was STZ-NA-induced DM mice treated with 1 % CMC-Na. Group 3 was STZ-NA-induced DM mice treated with 5 mg/kg glibenclamide as a positive control. Groups 4, 5 and 6 were STZ-NA-induced DM mice treated with 250, 500, and 1000 mg/kg HF344 extract in 1 % CMC-Na, respectively. All groups were subjected to a 12-h overnight fast before the experiment commenced. The mice were orally administered 2 g/kg of glucose, and 30 min later, the respective treatments were administered by oral gavage.

Following the above treatments in normal and DM mice, blood samples were collected from the tail vein at 0, 30, 60, 90, and 120 min after glucose administration. Blood glucose levels were measured using a glucose meter (Accu-Chek®, Roche). At the end of these OGTT experiments, the mice were sacrificed with carbon dioxide (CO_2) inhalation. Oral glucose tolerance curves for normal and DM mice were determined by plotting the changes in fasting blood glucose (FBG) concentrations (mg/dL) relative to the baseline against time. The area under the curve (AUC) over time was calculated using the linear trapezoidal method, as shown in Equation (2) [41]:

$$AUC = \frac{[C_0 + C_{30}] \cdot [t_{30} - t_0]}{2} + \frac{[C_{30} + C_{60}] \cdot [t_{60} - t_{30}]}{2} + \frac{[C_{60} + C_{90}] \cdot [t_{90} - t_{60}]}{2} + \frac{[C_{90} + C_{120}] \cdot [t_{120} - t_{90}]}{2} \quad \text{(Eq. 2)}$$

where C_0, C_{30}, C_{60}, C_{90}, and C_{120} represent the changes in FBG concentrations (mg/dL) relative to baseline at times t_0 (0 min), t_{30} (30 min), t_{60} (60 min), t_{90} (90 min), and t_{120} (120 min), respectively.

2.3.5.5. Continuous oral administration of HF344 extract. The antihyperglycemic effect of HF344 extract was assessed in STZ-NA-induced DM mice after daily oral administration for 28 days [41]. Mice were separated into six groups. Groups 1 and 2 were normoglycemic and STZ-NA-induced DM mice, receiving 1 % CMC-Na, as the normal and negative control groups, respectively. Group 3, the positive control group, included STZ-NA-induced DM mice treated with 5 mg/kg glibenclamide. Groups 4, 5, and 6 were STZ-NA-induced DM mice dosed with 250, 500, and 1000 mg/kg HF344 extract, respectively.

Each animal group was monitored for daily body weights and average food consumption. Blood samples were collected from the tail vein of fasted mice at the beginning (day 0), day 14, and day 28, and blood glucose levels were measured using a glucometer (Accu-Chek®, Roche). At the end of the study, the mice were sacrificed by CO_2 inhalation, and whole blood was collected via cardiac puncture. Moreover, gastrocnemius muscle was immediately harvested and rinsed three times in ice-cold normal saline. The collected organs were minced into small pieces and homogenized in ice-cold PBS (0.01 M, pH 7.4). The obtained homogenates were centrifuged at 5000 rpm for 15 min at 4 °C, then the supernatant was collected and used for the subsequent studies. The total protein content of homogenates muscle was determined by Bradford protein assay kit (Sigma-Aldrich, St. Louis, MO). The obtained blood samples were centrifuged at 3000 g for 10 min, yielding plasma, which was stored at −20 °C for later analysis of serum insulin levels. The serum insulin concentration was measured using a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Fujifilm Wako, Osaka, Japan), following the manufacturer's instructions. FBG and serum insulin levels on day 28 in each mouse were used to calculate the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) using the formula shown in Equation (3) [42,43]:

$$\text{HOMA-IR} = \frac{\text{fasting insulin } (\mu\text{IU/mL}) \times \text{fasting glucose } (\text{mg/dL})}{405} \quad \text{(Eq. 3)}$$

2.4. Antioxidative activities of HF344 extract in vitro and ex vivo

2.4.1. Total phenolic content and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays in vitro

The total phenolic content in the HF344 extract was quantified using the Folin-Ciocalteau method with some modifications [44]. An aliquot of the extract samples (20 μL) was mixed with 50 μL of Folin-Ciocalteu reagent, which had been diluted in a 1:4 ratio with distilled water. After 3 min, 150 μL of 7.5 % (w/v) Na_2CO_3 solution was added, and the mixture was shaken for 1 min. After incubating in darkness at room temperature for 120 min, the absorbance of the samples was measured at 765 nm using a microplate reader (Tecan, Switzerland). Gallic acid, at concentrations ranging 7.8–1000 μg/mL, was employed to generate a standard calibration curve. All assays were performed in triplicate. Total phenolic content was expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

The radical scavenging activity of HF344 extract against DPPH was determined using a previously described method [44]. Briefly, HF344 extracts at varying concentrations (1–100 μg/mL) dissolved in DMSO were mixed with a freshly prepared DPPH solution in methanol. The mixtures were allowed to react at room temperature in the dark for 30 min. The absorbance of the reaction mixtures was then measured at 515 nm using a microplate reader (Tecan, Switzerland). Ascorbic acid served as a positive control. Each assay was performed in triplicate, and the percentage of inhibition was expressed as follows:

$$\% \text{ inhibition} = \frac{(A_C - A_S)}{A_S} \times 100 \quad \text{(Eq. 4)}$$

where Ac and As are the absorbances of the control and the sample, respectively.

Then, the IC50 value of HF344's radical scavenging activity was calculated by plotting the percentage of inhibition against the log concentration of the extract.

2.4.2. Malondialdehyde (MDA) production in vitro

The amount of MDA, a major byproduct of lipid peroxidation during oxidative stress, was measured using a previous method [41] with slight modifications. A mixture of 1 mL HF344 extract (1–100 μg/mL) or Trolox (as a positive control), 4 mL of 99.5 % ethanol, 4.1 mL of 2.5 % linoleic acid in 99.5 % ethanol, 8.0 mL of 0.02 M phosphate buffer (pH 7.0), and 3.9 mL of distilled water was incubated in the dark at 40 °C for 10 days. After incubation, a mixture of 2 mL of 20 % trichloroacetic acid (TCA) and 2 mL of 0.67 % thiobarbituric acid (TBA) was added to 1 mL of the reaction mixture. The resulting mixture was boiled for 10 min and then rapidly cooled under running tap water. Then, the mixture was centrifuged at 5000 rpm for 5 min to separate the precipitate, and the absorbance of the supernatant was measured at 532 nm. The percentage of HF344's inhibitory activity on MDA production was calculated as follows [41]:

$$\text{Inhibition of MDA production} = \left[\frac{A_c - A_s}{A_c}\right] \times 100 \quad \text{(Eq. 5)}$$

where A_c and A_s are the absorbance of the control and sample, respectively.

The MDA levels were quantified as MDA equivalents (mg MDA/g linoleic acid) using a standard curve constructed by plotting the concentration of 1,1,3,3-tetramethoxypropane (TMP) (0.2–10.0 μg/mL), an MDA precursor, against its corresponding absorbance. The IC50 value was calculated from the plots of percentage inhibition of MDA production versus the extract's concentration.

2.4.3. MDA production in homogenated muscle ex vivo

The effect of HF344 extract on oxidative stress was determined by measuring the MDA production *ex vivo* in the homogenated muscle collected from DM mice treated with the extract for 28 days, as previously described [41] with few modifications. The muscle homogenate (300 μL) was reacted with 600 μL of 40 % TCA and 600 μL of 0.67 % TBA. The mixture was heated to 95–100 °C for 30 min, then cooled under running tap water to halt the reaction. The solution was then centrifuged at 5000 rpm for 15 min at 4 °C to separate the supernatant. The absorbance of the supernatant was measured at 532 nm to quantify the MDA content. To determine MDA concentration, a standard curve was generated using 1,1,3,3-tetramethoxypropane (TMP) as the MDA precursor. The final MDA concentration in the muscle homogenate was expressed in ng/mg protein.

2.4.4. Effects of HF344 extract on STZ-induced oxidative stress in vitro

The effect of the HF344 extract on STZ-induced oxidative stress was investigated using the rat insulinoma cell line RINm5F (CRL-2058™), obtained from ATCC® (USA). STZ is known to induce oxidative stress and subsequent cytotoxicity in RINm5F cells, which serve as a cell culture model for pancreatic β-cells [45]. Initially, RINm5F cells were seeded in 96-well plates at a density of 1×10^5 cells/mL. After a 24-h incubation period, the cell culture medium was substituted with HF344 extract at concentrations of 1, 10, or 100 μg/mL. Following an additional 24-h incubation, the medium containing the extract was replaced with STZ solution at a concentration of 6 mM in each well, and the cells were further incubated for 24 h at 37 °C. Subsequently, an MTT solution (5 mg/mL in PBS) was introduced to each well, and the cells were subjected to an additional 4-h incubation at 37 °C. Following this incubation, formazan crystals were dissolved by the addition of 100 μL of DMSO to each well, and absorbance was measured at 550 and 595 nm using a microplate reader (Tecan, Switzerland). Cell viability was computed using a formula (Eq. (1)), and a concentration-response curve was constructed by plotting cell viability against the concentrations of HF344 extract.

In a separate experimental setup where the treatment order of STZ and HF344 extract was reversed, RINm5F cells were seeded in 96-well plates at a density of 1×10^5 cells/mL. After a 24-h incubation period, the cell culture medium was exchanged with STZ solution at a concentration of 6 mM in each well, and the cells were incubated for 1 h at 37 °C. Following this incubation, the STZ-containing medium was substituted with HF344 extract at concentrations of 1, 10, and 100 μg/mL, and the cells were further cultured for 24 h under standard conditions (37 °C, 5 % CO_2, 95 % relative humidity). After this incubation period, the MTT assay was conducted following the previously described protocol.

2.5. Statistical analyses

The data from *in vitro* studies were presented as the mean ± standard deviation (SD), while the data from *in vivo* studies were presented as the mean ± standard error of mean (SEM). The significant differences were analyzed using one-way ANOVA, followed by post-hoc Tukey's multiple comparison. Statistical testing was conducted using SPSS version 18 (SPSS Inc., Chicago, IL, USA). The statistical significance level was set at $p < 0.05$.

3. Results

3.1. Quantitative phytochemical analysis

The phytochemical profile of HF344 was analyzed using UHPLC-DAD-Q-Orbitrap-MS. A representative chromatogram is shown in Fig. 1. Identification of peaks was performed using mass spectral data compared with the National Institute of Standards and Technology (NIST) and mzCloud database. The results are demonstrated in Table 3. The major components contained in HF344 extract, tentatively identified using mass spectrum, were methyl 15-(benzoyloxy)-2,11,12-trihydroxy-3,16-dioxo-13,20-epoxypicrasa-1,4-dien-21-oate (**4**) and piperine (**6**) (Fig. 1A-C). Minor components included gallic acid (**1**), myricetin 3-O-β-D-galactopyranoside (**2**), and ellagic acid (**5**), along with some unidentified compounds. Confirmation of gallic acid (**1**) and piperine (**6**) was conducted based on co-chromatography with their authentic standards. Compound **3** predominantly exhibited the molecular formula $C_{19}H_{23}O_9$, as determined by $[M+H]^+$ at *m/z* 395.1335 (calculated 395.13366) and $[M-H]^-$ at *m/z* 393.1186 (calculated 393.11911); however, it remained unidentified within our database. For further identification of compound **3**, its UV and MS spectra with fragmentation were provided in supplementary data.

As shown in Fig. 2, the HPLC chromatogram of the HF344 extract exhibited a prominent peak at a retention time of 5.89 ± 0.06 min (Fig. 2B), corresponding to the piperine standard (Fig. 2A). The content of piperine in the HF344 extract was 0.68 ± 0.03 % w/w.

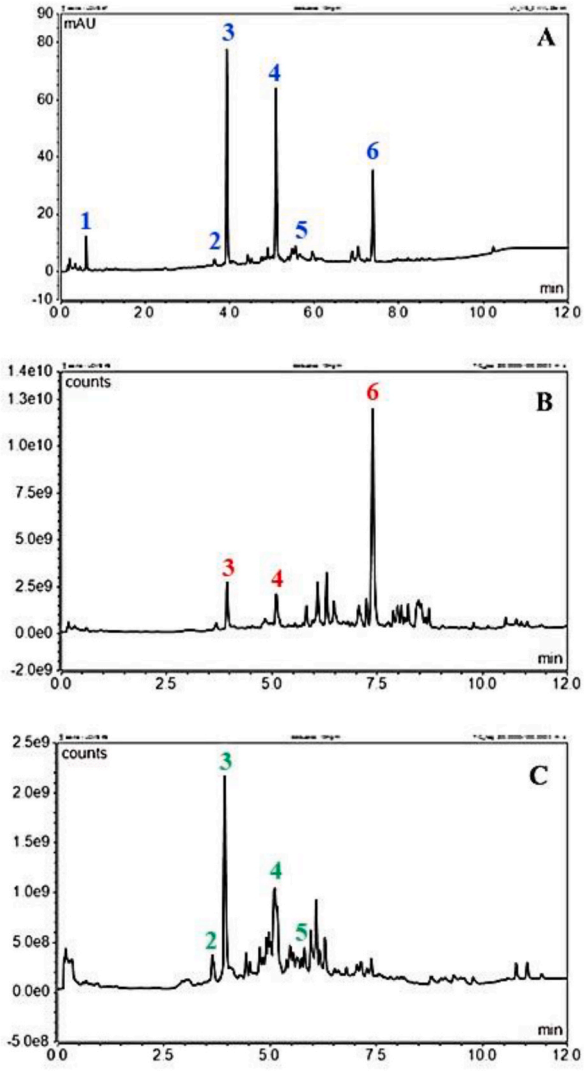

Fig. 1. UHPLC chromatograms of HF344 extract at a concentration of 10 mg/mL, detected at UV 254 nm (A), full scan (200–1000 *m/z*) with positive mode (B), and full scan (200–1000 *m/z*) with negative mode (C).

Table 3
Identification of major components in HF 344 extract by UHPLC-DAD-Q-Orbitrap-MS.

Peak No.	Retention time (min)	Mode	Base peak (*m/z*)	**Results**
1	0.60	–	–	Gallic acid[a]
2	3.65	Negative	479.0825	Myricetin 3-*O*-β-*D*-galactopyranoside $[M-H]^-$ calculated for $C_{21}H_{20}O_{13}$ = 479.08311
3	3.93	Positive	395.1335	Unidentified compound with molecular formula $C_{19}H_{23}O_9$ $[M+H]^+$ calculated for $C_{19}H_{23}O_9$ = 395.13366
		Negative	393.1186	$[M-H]^-$ calculated for $C_{19}H_{23}O_9$ = 393.11911
4	5.09	Positive	541.1702	Methyl 15-(benoyloxy)-2,11,12-trihydroxy-3,16-dioxo-13,20-epoxypicrasa-1,4-dien-21-oate or its isomer $[M+H]^+$ calculated for $C_{28}H_{28}O_{11}$ = 541.17044
		Negative	539.1552	$[M-H]^-$ calculated for $C_{28}H_{28}O_{11}$ = 539.15588
5	5.58	Negative	300.9989	Ellagic acid $[M-H]^-$ calculated for $C_{14}H_6O_8$ = 300.99899
6	7.38	Positive	286.1435	Piperine[a] $[M-H]^-$ calculated for $C_{17}H_{19}NO_3$ = 286.14377

[a] Gallic acid and piperine were also identified by co-chromatography with standards.

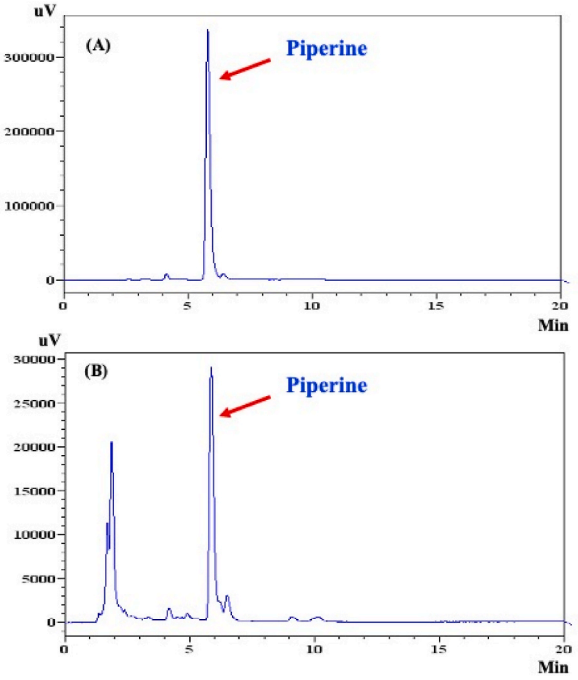

Fig. 2. HPLC chromatograms of piperine standard (A) and HF344 extract (B), detected at the wavelength of 341 nm.

3.2. Antihyperglycemic activities of HF344 extract in vitro and ex vivo

3.2.1. Insulin secretion stimulatory assay

As shown in Fig. 3A, HF344 extract at the concentrations of 1–1000 μg/mL did not affect the viability of RINm5F cells when compared to the control, indicating no toxic effect on the cells. Therefore, the HF344 concentration range of 1–100 μg/mL was selected for further determination of insulin secretion in RINm5F cells in subsequent experiments. Fig. 3B shows the average insulin secretion in RINm5F cells treated with HF344 extract, compared to the untreated control. Glibenclamide (10 μg/mL), used as a positive control, produced a significantly increased insulin secretion of approximately 4-fold, compared to the control ($p < 0.01$). HF344 extract increased insulin secretion in RINm5F cells compared to the control ($p < 0.05$), in a concentration-dependent manner.

3.2.2. Glucose uptake in everted sacs of the small intestine ex vivo

As depicted in Fig. 4, HF344 extract demonstrated a concentration-dependent inhibition of glucose uptake in everted sacs of the mouse intestine *ex vivo*. Concentrations of 1.25, 2.5, 5, and 10 mg/mL resulted in reductions of 9.1 %, 21.3 %, 26.3 %, and 39 %, respectively, compared to the control. NaF, a known inhibitor of intestinal glucose absorption, caused a significant 34.6 % reduction in glucose uptake.

3.2.3. mRNA expressions of GLUT1 and GLUT4 in myoblasts in vitro

As shown in Fig. 5A and B, HF344 extract at concentrations of 31.25–1000 μg/mL did not affect the viability of L6 skeletal

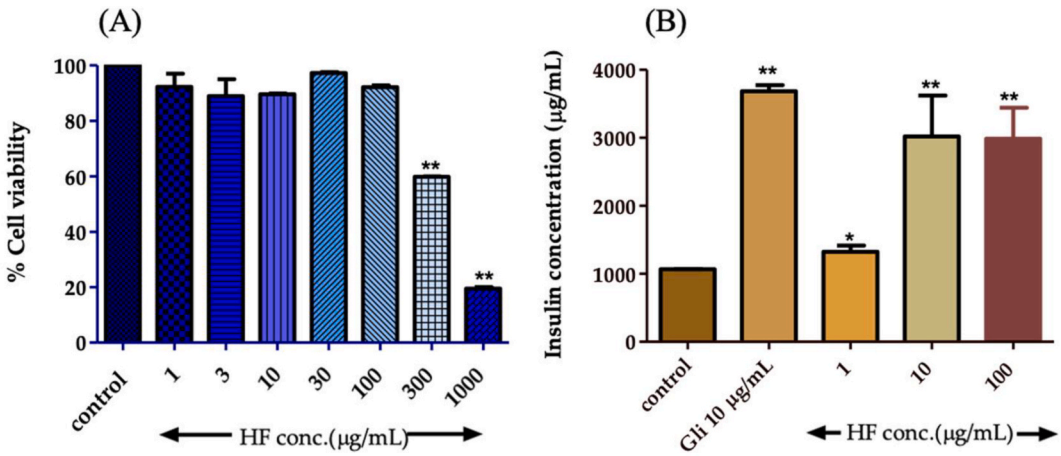

Fig. 3. Effect of HF344 extract on cell viability (A) and insulin secretion in the rat insulinoma cell line RINm5F (B). Data are expressed as the mean ± SD (n = 3). *·**p < 0.05 and p < 0.01 vs. control, respectively; Gli: glibenclamide; HF: HF344.

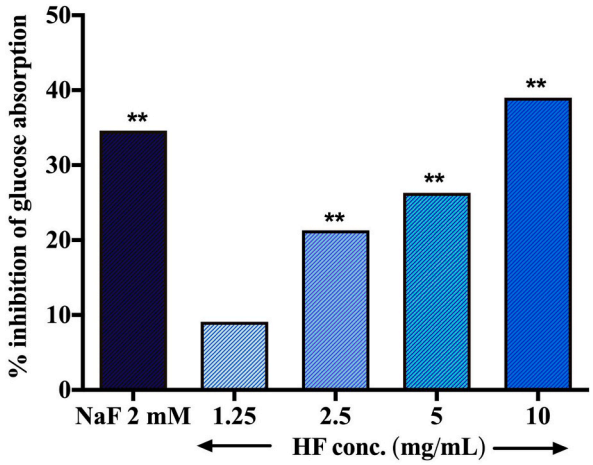

Fig. 4. Inhibitory effect of HF344 extract on glucose uptake in the everted mouse intestinal sac *ex vivo* compared with control. Data are expressed as the mean ± SD (n = 3). **p < 0.01 vs. control, HF: HF344.

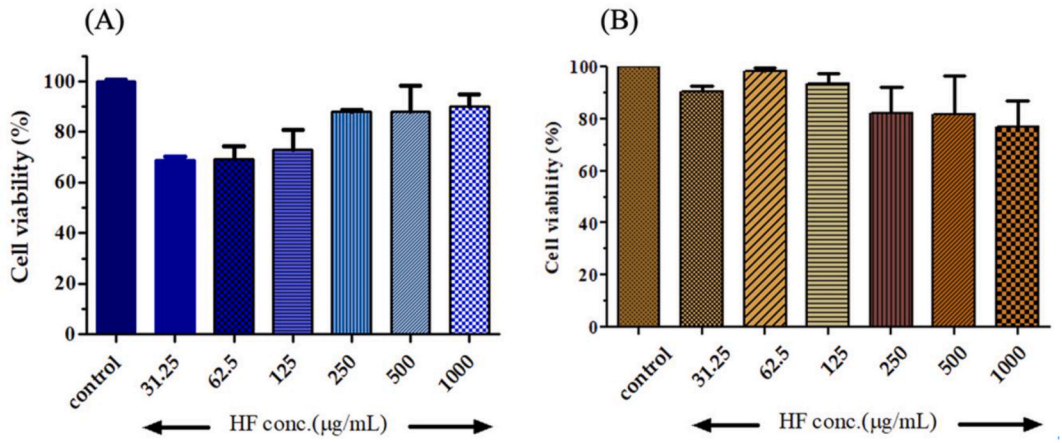

Fig. 5. Effects of HF344 extract on the cell viability of H9c2 cardiomyoblasts (A) and L6 skeletal myoblasts (B). Data are expressed as the mean ± SD (n = 3). HF: HF344.

myoblasts and H9c2 cardiomyoblasts when compared to a control, indicating no toxic effect on the cells. Therefore, HF344 extract concentrations of 100 and 200 μg/mL were selected for further determination of mRNA expression of GLUT1 and GLUT4 in both cells.

Fig. 6A-D demonstrate that HF344 extract upregulated the mRNA expression of GLUT1 in L6 cells while exhibiting no significant effect on GLUT1 expression in H9c2 cells and GLUT4 expression in either cell type. This suggests that HF344 extract may modulate the abundance of GLUT1, potentially enhancing glucose uptake into muscle cells. Treatment of L6 cells with HF344 extract showed a concentration-dependent increase in GLUT1 mRNA expression, with the most pronounced effect observed at a concentration of 200 μg/mL, indicating a potential role of HF344 extract in augmenting GLUT1 levels and subsequent glucose uptake into skeletal muscle. However, concentrations of HF344 up to 200 μg/mL did not elicit any significant alteration in GLUT1 mRNA expression in L6 cells. Moreover, treatment with HF344 extract at concentrations ranging from 100 to 200 μg/mL did not induce the synthesis of GLUT1 and GLUT4 in H9c2 cardiomyoblasts.

3.2.4. α-Glucosidase activity in vitro

In vitro assessment of α-glucosidase activity revealed a concentration-dependent inhibition by HF344 extract, surpassing the inhibitory effect observed with acarbose (as a positive control). The IC50 values for HF344 extract and acarbose were determined as 96.74 ± 0.29 μg/mL and 511.0 ± 16.8 μg/mL, respectively.

3.2.5. Antihyperglycemic activities of HF344 extract in vivo

3.2.5.1. Acute oral toxicity test. Oral administration of HF344 extract at a dose of 2000 mg/kg did not induce any discernible signs of toxicity or alterations in behavior compared to the control group. Additionally, no mortality occurred among the mice over the 14-day observation period. Throughout the study, the body weights of all mice remained relatively constant, with no significant differences

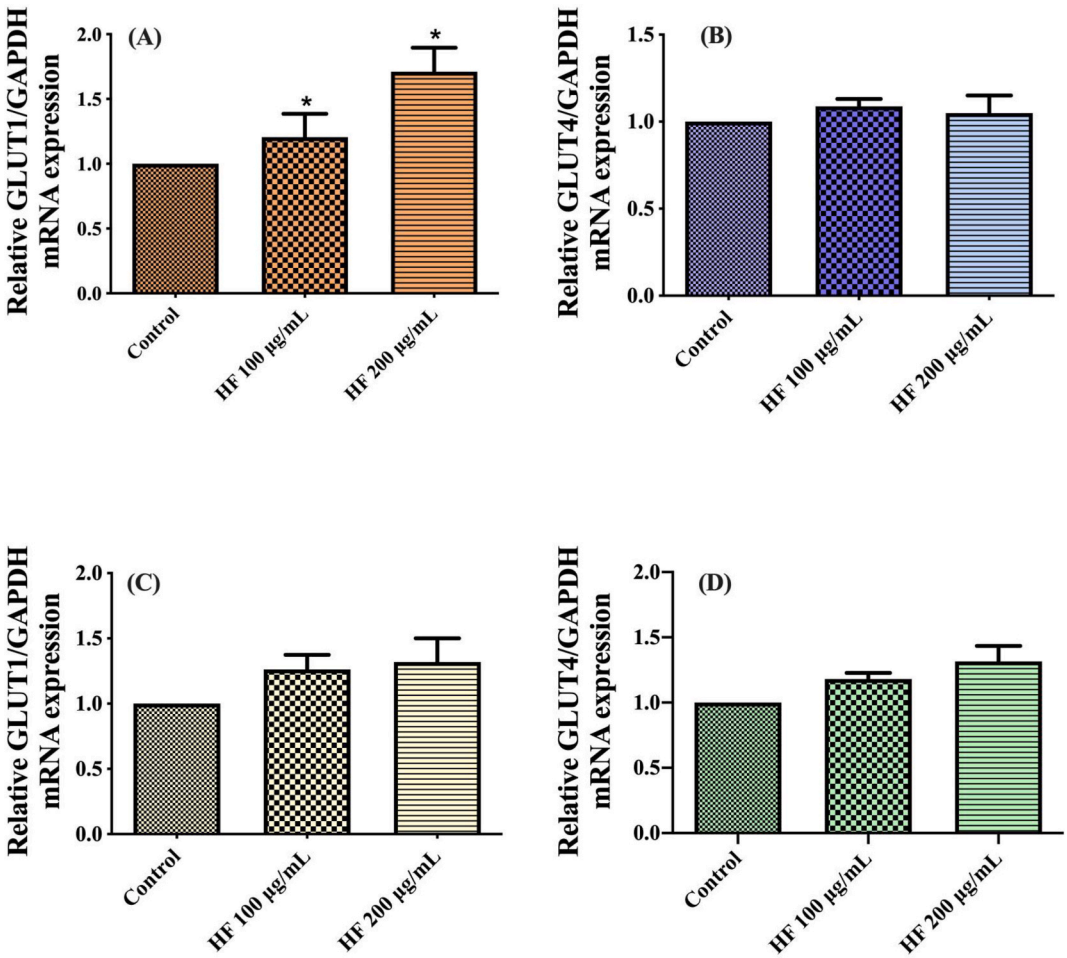

Fig. 6. Effects of HF344 on mRNA expressions of GLUT1 (A) and GLUT 4 (B) in L6 skeletal myoblasts and on GLUT1 (C) and GLUT 4 (D) in H9c2 cardiomyoblasts. Data are expressed as the mean ± SD (n = 4). *p < 0.05 vs control. HF: HF344; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

observed between the control group and the HF344 extract-treated group. These results indicate, in accordance with OECD toxicity test guideline 425, that the LD50 value of HF344 exceeds 2000 mg/kg.

3.2.5.2. Oral glucose tolerance tests. Fig. 7A and B shows the changes in FBG concentrations relative to the baseline value measured at various time points (0–120 min) following the oral administration of glucose in normoglycemic and STZ-NA-induced DM mice, respectively. In normal mice treated with HF344 extract at doses of 250, 500, and 1000 mg/kg, the area under the FBG-time curve (AUC) did not significantly differ from that of the normal control group, as shown in Fig. 7C. As depicted in Fig. 7B, both glibenclamide (5 mg/kg) and HF344 extract (1000 mg/kg) treatment led to significant reductions in FBG levels at 30, 60, 90, and 120 min compared to the untreated diabetic group ($p < 0.05$). Evaluation of the AUC presented in Fig. 7D indicated that oral administration of HF344 extract at doses of 500 and 1000 mg/kg exhibited antihyperglycemic effects similar to those of glibenclamide.

3.2.5.3. Continuous oral administration of HF344 extract. As shown in Table 4, after 28 days of daily oral administration of HF344 extract, untreated DM mice exhibited significant changes in body weight (decrease) and food intake (increase) compared to the normal control mice ($p < 0.05$), indicating symptoms of diabetes. Similar trends in body weight reduction and food intake elevation were observed in T2DM mice treated with glibenclamide and HF344 extract; however, none of these treatments significantly influenced these parameters.

FBG levels in STZ-NA-induced DM mice were markedly elevated compared to those in the normal control group ($p < 0.05$), steadily increasing throughout the study period, as illustrated in Fig. 8. On the 14th day of treatment, administration of 5 mg/kg glibenclamide and 1000 mg/kg HF344 extract resulted in significant reductions in FBG levels in STZ-NA-induced DM mice by 32.40 % and 31.09 %, respectively ($p < 0.05$). By the end of the 28-day treatment period, the group treated with 1000 mg/kg HF344 exhibited a substantial 30.83 % reduction in FBG ($p < 0.05$), while the positive control, 5 mg/kg glibenclamide, demonstrated a significant reduction of 35.03 % in FBG in STZ-NA-induced DM mice ($p < 0.05$). Conversely, doses of 250 and 500 mg/kg HF344 did not yield significant effects on FBG in DM mice compared to the untreated diabetic group. Notably, the effect of HF344 was observed to be dose-dependent.

Following 28 days of treatment, the diabetic group exhibited a notable reduction in serum insulin levels compared to the normal control group ($p < 0.05$), as shown in Fig. 9. Subsequent administration of glibenclamide (5 mg/kg) and HF344 extract (1000 mg/kg) resulted in significant increases in serum insulin levels compared to the diabetic group ($p < 0.05$). However, the insulin levels of the T2DM group treated with 250 and 500 mg/kg of HF344 extract did not show significant differences from those of the diabetic group.

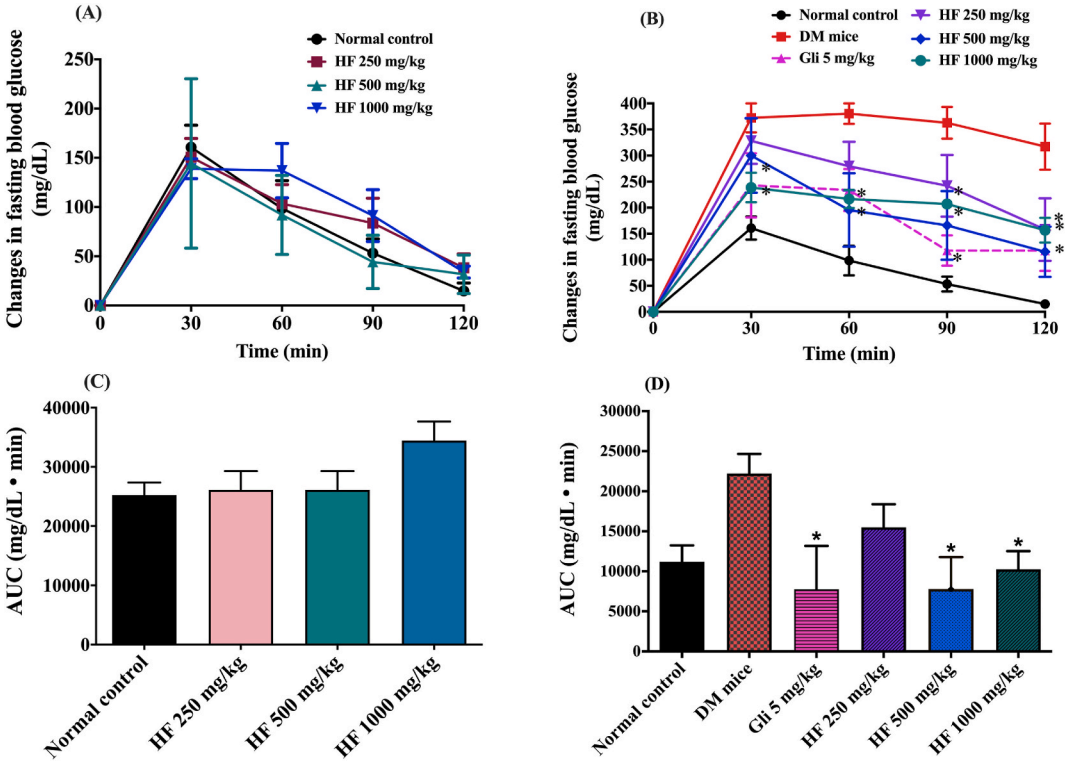

Fig. 7. Effects of HF344 extract on the changes in FBG levels relative to the baseline in normal (A) and DM mice (B) and area under the FBG-time curve (AUC) of normal (C) and DM mice (D) in oral glucose tolerance test. Data are expressed as the mean ± SEM (n = 6–8/group). #$p < 0.05$ vs. NC, *$p < 0.05$ vs. DM; NC: Normal Control; DM: Diabetes mellitus; Gli 5: DM + glibenclamide (5 mg/kg); HF 250 mg/kg: DM + HF344 (250 mg/kg); HF 500 mg/kg: DM + HF344 (500 mg/kg); HF 1000 mg/kg: DM + HF344 (1000 mg/kg).

Table 4
Effects of 28-day oral administration of HF344 extract on body weight and food intake in STZ-NA-induced DM mice.

Groups	Baseline		Day 14		Day 28	
	Bodyweight (g)	Food intake (g)	Bodyweight (g)	Food intake (g)	Bodyweight (g)	Food intake (g)
Normal control	40.45 ± 0.92	4.83 ± 0.10	40.40 ± 0.95	4.62 ± 0.02	41.95 ± 0.90	4.00 ± 0.10
Diabetic control	32.82 ± 1.13$^{\#}$	6.88 ± 0.66$^{\#}$	33.35 ± 0.95$^{\#}$	6.48 ± 0.12$^{\#}$	33.81 ± 1.24$^{\#}$	7.25 ± 0.51$^{\#}$
Glibenclamide (5 mg/kg)	34.34 ± 1.77	7.15 ± 0.50	34.42 ± 2.23	6.56 ± 0.09	36.11 ± 2.06	7.40 ± 0.55
HF344 (250 mg/kg)	36.02 ± 1.73	7.83 ± 0.50	36.07 ± 1.80	7.25 ± 0.07	36.21 ± 2.05	7.79 ± 0.45
HF344 (500 mg/kg)	33.80 ± 2.18	6.85 ± 0.29	33.32 ± 2.16	6.31 ± 0.56	33.77 ± 2.42	7.51 ± 0.44
HF344 (1000 mg/kg)	35.81 ± 1.23	6.64 ± 0.46	34.96 ± 1.40	6.40 ± 0.42	36.07 ± 1.21	7.32 ± 0.46

Data are expressed as the mean ± SEM (n = 6–8). HF: HF344, #p < 0.05 *vs* normal control.

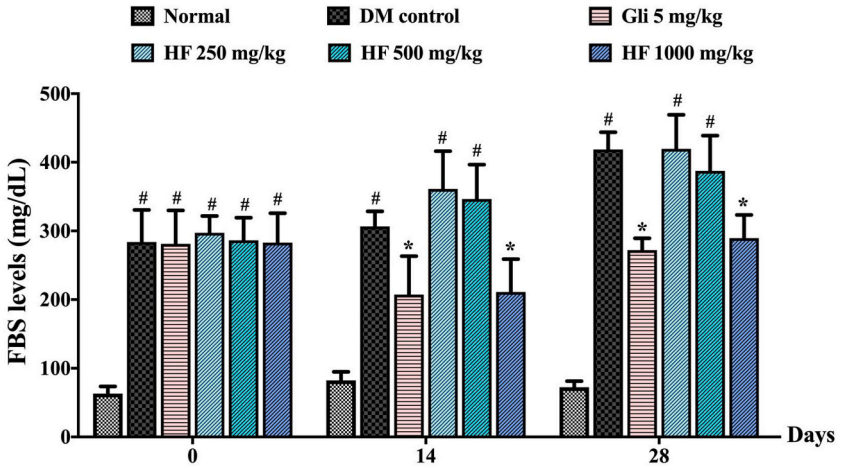

Fig. 8. Effect of HF344 extract on FBG after oral treatment for 28 days. Data are expressed as the mean ± SEM (n = 6–8), #p < 0.05 *vs* normal control, *p < 0.05 *vs* DM control, Gli 5 mg/kg: DM + glibenclamide 5 mg/kg; HF 250 mg/kg: DM + HF344 (250 mg/kg); HF 500 mg/kg: DM + HF344 (500 mg/kg); HF 1000 mg/kg: DM + HF344 (1000 mg/kg).

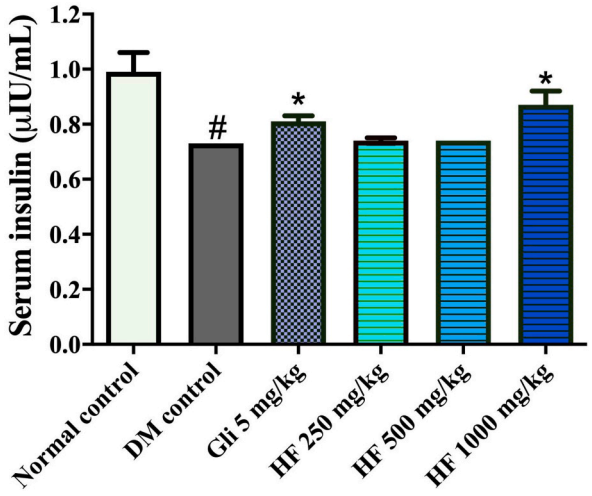

Fig. 9. Effect of HF344 extract on serum insulin levels after oral treatment for 28 days. Data are expressed as the mean ± SEM (n = 6–8), #p < 0.05 *vs* normal control, *p < 0.05 *vs* DM control, Gli 5 mg/kg: DM + glibenclamide 5 mg/kg; HF 250 mg/kg: DM + HF344 (250 mg/kg); HF 500 mg/kg: DM + HF344 (500 mg/kg); HF 1000 mg/kg: DM + HF344 (1000 mg/kg).

The induction of T2DM in experimental mice led to a noteworthy elevation in HOMA-IR compared to the normal control group (p < 0.05). Treatment with glibenclamide (5 mg/kg) and HF344 extract (1000 mg/kg) resulted in significant reductions in HOMA-IR levels in T2DM mice. However, T2DM mice treated with HF344 extract at doses of 250 and 500 mg/kg did not exhibit a significant

decrease in HOMA-IR compared to the untreated diabetic group, as illustrated in Fig. 10.

3.3. Antioxidative activities of HF 344 extract in vitro and ex vivo

3.3.1. Total phenolic content and DPPH assays

Table 5 presents the total phenolic content in the HF344 extract, as well as the IC50 values obtained from DPPH assays for HF344 extract and ascorbic acid.

3.3.2. MDA production in vitro

The inhibitory effect of HF344 extract on MDA generation was concentration-dependent. The percentage inhibition of MDA production varied from 16.4 ± 3.97 % to 85.5 ± 2.74 % across HF344 extract concentrations ranging from 4 to 16 μg/mL. The IC50 of Trolox, as a positive control, was determined to be 0.15 ± 0.04 μg/mL, while that of HF344 extract was 7.24 ± 0.46 μg/mL.

3.3.3. MDA production in homogenated muscle ex vivo

As presented in Fig. 11, the DM control group exhibited a significant increase in MDA levels compared to the normal control group ($p < 0.05$). Treatment with HF344 extract decreased MDA production in the homogenized muscle; however, there was no significant effect compared to DM mice.

3.3.4. Effects of HF344 extract on STZ-induced oxidative stress in vitro

The modulating effects of HF344 on the STZ-induced cytotoxicity were examined using the rat pancreatic cell line, RINm5F. As shown in Fig. 12A and B, the exposure of RINm5F cells to STZ (6 mM) markedly reduced the viability of RINm5F cells, approximately by 50 %. However, pretreatment with HF344 at concentrations of 1, 10, and 100 μM for 1 h led to a notable recovery in cell viability, approximately by 65 %, 62 %, and 64 %, respectively, in comparison to STZ-induced oxidative stress in RINm5F cells (Fig. 12B). Nonetheless, no significant protective effect of HF344 on cell viability was observed when compared with the STZ-induced oxidative stress in RINm5F cells (Fig. 12A).

4. Discussion

In recent years, numerous Thai herbal formulations, incorporating traditional herbal ingredients, have emerged as commercially available natural medicinal preparations utilized for managing T2DM within local markets. However, approximately 30 % of medicinal plants used in Thailand for treating diabetes still lack experimental evidence to validate claims regarding the mechanisms of action and the phytochemicals responsible for antidiabetic properties [4]. Since HF344 extract was formulated to comprise fifteen ingredients potentially beneficial for alleviating DM complications and symptoms, we conducted this study to elucidate the pharmacological effects of HF344 extract under *in vitro*, *ex vivo*, and *in vivo* conditions, aiming to provide mechanistic insights into its therapeutic potential in managing T2DM. Through a comprehensive array of experiments, we successfully elucidated the multifaceted effects of HF344 extract on glucose metabolism and insulin regulation. *In vitro* investigations revealed that HF344 exerts its antihyperglycemic effects through various mechanisms, aligning with findings observed in STZ-NA-induced DM mice, a well-established model of T2DM. Following consecutive oral administration in STZ-NA-induced DM mice, HF344 extract (1000 mg/kg) significantly

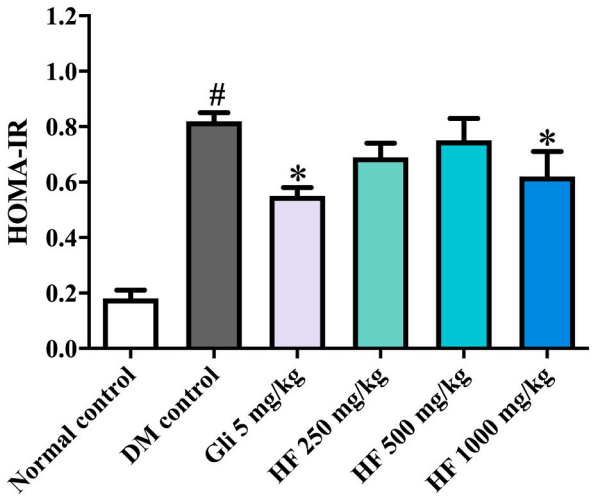

Fig. 10. Effect of HF344 extract on HOMA-IR levels after oral treatment for 28 days. Data are expressed as the mean ± SEM (n = 6–8), #$p < 0.05$ *vs* normal control, *$p < 0.05$ *vs* DM control, Gli 5 mg/kg: DM + glibenclamide 5 mg/kg; HF 250 mg/kg: DM + HF344 (250 mg/kg); HF 500 mg/kg: DM + HF344 (500 mg/kg); HF 1000 mg/kg: DM + HF344 (1000 mg/kg).

Table 5
Total phenolic content and IC50 values for DPPH assays.

	Total phenolic content [mg GAE/g extract]	DPPH assay IC50 [μg/mL]
HF344 extract	38.11 ± 5.38	34.99 ± 4.15
Ascorbic acid	N/A	6.47 ± 0.54

Data are expressed as the mean ± SD (n = 4). GAE: gallic acid equivalent; IC50: 50 % inhibitory concentration; DPPH: 2,2-diphenyl-1-picrylhydrazyl; N/A: not applied for ascorbic acid.

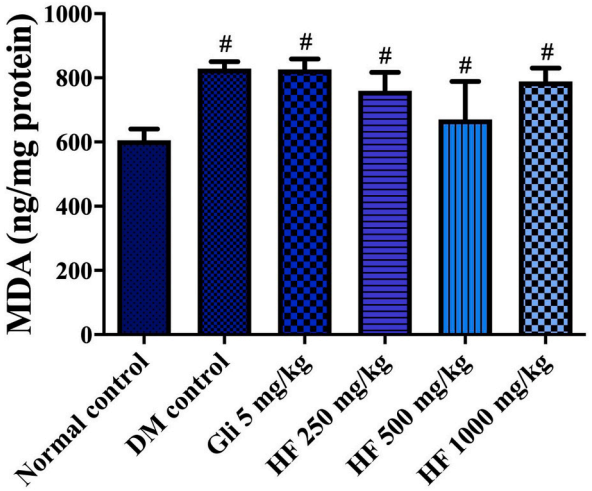

Fig. 11. Effect of HF344 extract on MDA levels on homogenate muscle. Data are expressed as the mean ± SEM (n = 6–8), #p < 0.05 *vs* normal control, *p < 0.05 *vs* DM control, Gli 5 mg/kg: DM + glibenclamide 5 mg/kg; HF 250 mg/kg: DM + HF344 (250 mg/kg); HF 500 mg/kg: DM + HF344 (500 mg/kg); HF 1000 mg/kg: DM + HF344 (1000 mg/kg).

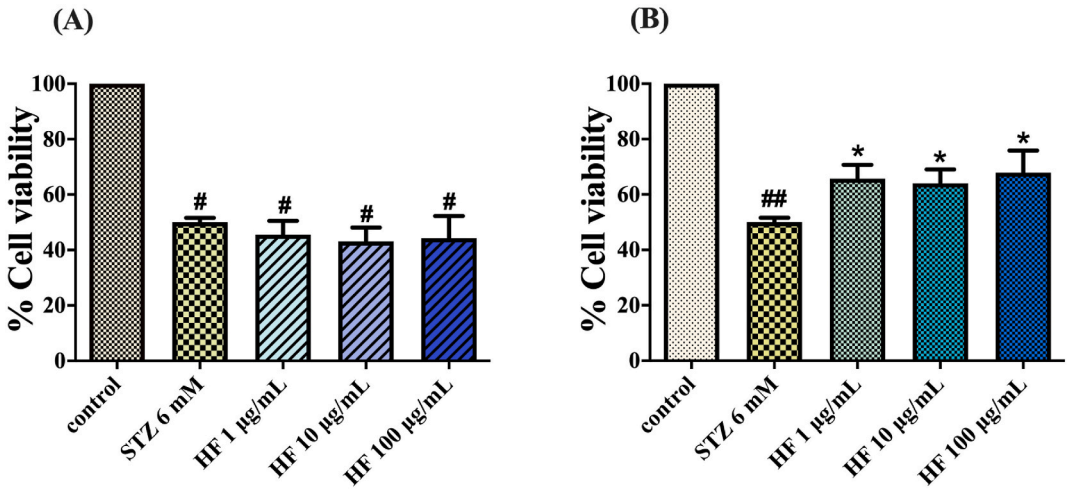

Fig. 12. Effects of HF344 extract on the cell viability of the STZ-treated RINm5F cells, assessed using the MTT assay. In the "protective" condition (A), cell viability was evaluated after incubation with HF344 extract (1, 10, and 100 μg/mL) for 1 h before the addition of 6 mM STZ. In the "recovery" condition (B), cell viability was assessed after the addition of 6 mM STZ followed by incubation with HF344 extract (1, 10, and 100 μg/mL). Data are expressed as the mean ± SD (n = 4), #p < 0.05 *vs* control; *p < 0.05 *vs* STZ-treated cells, STZ, streptozotocin; HF: HF344.

decreased FBG after 14 and 28 days, and increased serum insulin levels. Of special note is the fact that the antihyperglycemic efficacy of HF344 extract was comparable to that of glibenclamide, a commonly used antidiabetic medicine.

The present study provided *in vitro, ex vivo,* and *in vivo* mechanistic insights into the hypoglycemic effects of HF344 extract, by employing various methodologies to evaluate the pharmacological and biochemical effects of the polyherbal extract. GLUT1 serves pivotal roles in regulating glucose metabolism and cellular functions in muscle tissues [46]. The observed antihyperglycemic effects of

HF344 *in vivo* may be attributed, at least partially, to the observed upregulation of GLUT1 mRNA levels by HF344 extract, as demonstrated *in vitro* using L6 skeletal myoblasts. This upregulation of GLUT1 potentially facilitates increased glucose uptake into skeletal muscle cells, thereby contributing to enhanced overall glucose clearance and improved insulin sensitivity. Although HF344 extract did not significantly change GLUT1 expression in H9c2 cells or GLUT4 expression in either cell type, the observed GLUT1 upregulation suggests the potential of the extract to augment glucose uptake into muscle cells, indicating its influence on insulin-independent tissues. The notable increase in insulin secretion further underlies the efficacy of HF344 extract in lowering FBG levels, consequently leading to improved glycemic control, as also evidenced by the sustained treatment effect observed over 28 days in STZ-NA-induced DM mice. Moreover, oral administration of HF344 extract suggests a potential reduction in insulin resistance, as indicated by the observed decrease in HOMA-IR, a reliable measure for assessing insulin resistance [47].

There have been several emerging evidence revealing that hyperglycemia-induced oxidative stress plays a crucial role in the pathogenesis of T2DM and its associated complications. Chronic hyperglycemia initiates oxidative stress, culminating in lipid peroxidation, which subsequently leads to pancreatic β-cell dysfunction, insulin resistance, and the onset of various complications encompassing cardiovascular, renal, hepatic diseases, retinopathy, neuropathy, and gastrointestinal disorders. Antioxidants have been reported to protect pancreatic β-cells through the attenuation of both islet fibrosis and β-cell apoptosis in T2DM rats [48]. In this study, we used the STZ-NA model of type 2 diabetes, which is reported to be suitable for assessing the effectiveness of new potential anti-diabetic agents. This model is particularly advantageous for studying diabetes and its complications due to its characteristic features of stable hyperglycemia and glucose intolerance [49]. HF344 extract demonstrated protective properties through its antioxidative activity, as evidenced by its ability to inhibit the DPPH radicals and MDA production in TBARs assays. Moreover, HF344 extract demonstrated the capacity to enhance cell survival from STZ-induced cell death in RINm5F cells, a rat insulinoma cell line widely utilized as a model to study insulin secretion mechanisms and develop novel therapeutic approaches for diabetes [35]. The present results, obtained from experiments with RINm5F cells, suggest a modulatory potential of HF344 to restore pancreatic β-cell functions when the cells are damaged by oxidative stress. Integrated evidence from both *in vitro* and *in vivo* studies suggested that HF344 extract can boost insulin production and functionality.

Insulin is essential for promoting cellular glucose uptake by facilitating the translocation of the glucose transporter GLUT4 to the plasma membrane. Upon binding to the tyrosine kinase receptor, insulin initiates the activation of protein kinase B (PKB), also known as Akt, and protein kinase C, which subsequently accelerate the mobilization of GLUT4 from intracellular storage compartments to the cell surface [46]. In our *in vivo* study, we administered HF344 extract (1000 mg/kg) daily to STZ-NA-induced DM mice for 28 days. This intervention resulted in a significant decrease in FBG levels by approximately 30 %, accompanied by notable enhancements in serum insulin concentrations, compared to untreated DM mice. These findings suggest that HF344 may exert its effects through mechanisms involving enhanced insulin production and augmented facilitation of glucose uptake for energy metabolism, culminating in lowered glucose levels. The lack of changes in GLUT4 expression in the cell lines of skeletal muscle (L6) and cardiomyoblasts (H9c2), following exposure to HF344 extract, suggests that HF344 extract did not directly influence the GLUT4 gene transcription.

Furthermore, the present study employed the OGTT in a single-dose administration experiment to assess the hypoglycemic properties of HF344 extract in both normal and DM mice. Oral administration of HF344 (500 and 1000 mg/kg) significantly reduced the FBG levels and area under the FBG-time curve during the OGTT, indicating improved glucose tolerance and reduced hyperglycemia. The underlying mechanism of this effect may be elucidated by findings from *in vitro* and *ex vivo* experiments demonstrating concentration-dependent inhibitions of α-glucosidase activity and glucose uptake into everted small intestine sacs by HF344. These results imply a potential avenue for HF344 in mitigating postprandial hyperglycemia by impeding glucose flux into the bloodstream, partly through modulation of carbohydrate digestion and delayed glucose absorption, thereby mitigating hyperglycemia [47].

In this study, HF344 did not demonstrate antihyperglycemic effects in normoglycemic mice. Recent research identified adaptations in both functional and structural aspects among rats with T2DM, including an augmentation in the number of enterocytes within the villi of the small intestine. Consequently, T2DM rats exhibited an enhanced capacity for glucose absorption in the small intestine [48], leading to compromised glucose tolerance compared to normoglycemic rats. Hence, it is plausible that the inhibition of glucose absorption could be more pronounced in T2DM rats under hyperglycemic conditions compared to normoglycemic rats. This rationale was reportedly applicable to the antihyperglycemic investigations conducted on the specific composition of the HF344 formulation [10, 50]. Nonetheless, the absence of a hypoglycemic effect in healthy individuals may represent a desirable attribute of HF344's impact on glucose absorption in the small intestine, thereby offering potential benefits for glycemic control.

In this study, HF344 primarily exerts its antihyperglycemic effects through several mechanisms: inhibiting glucose absorption via α-glucosidase inhibition, stimulating insulin production, and upregulating the mRNA expression of the GLUT1 transporter. The therapeutic potential of HF344 is attributed to its complex blend of botanical ingredients, each contributing to the overall pharmacological activity. As shown in Table 1, numerous studies have documented the α-glucosidase inhibition activity of various components within the HF344 herbal formula, including *Allium sativum* [6–8], *Suregada multiflora* [9], *Acanthus polystachyus* [10], *Piper nigrum* L. [13–15], *Cleome viscosa* [16], *Cyperus rotundus* [18,19], *Terminalia chebula* [20,21], *Curcuma longa* [23–25], *Curcuma zedoaria* [26], *Senna alexandrina* [28], and *Smilax glabra* [30]. *Allium sativum* [6], *Terminalia chebula* [20], *Smilax glabra* [30], and *Terminalia arjuna* [33] have been shown to enhance insulin sensitivity and production in *in vivo* studies. Additionally, *Allium sativum* [6], *Terminalia chebula* [20], *Alpinia galanga* [22], and *Terminalia arjuna* [33] exhibits other antidiabetic mechanisms such as improvement of glycosylated hemoglobin, restoring the function of islet β cells, stimulating GLUT4 expression, decrease HOMR-IR, and increase SIRT1. Moreover, *Acanthus ebracteatus* has been reported to possess wound-healing effects [10], while *Cleome viscosa* L. exhibits immunomodulatory properties [16]. The combination of antioxidative and anti-inflammatory effects from certain herbs in the HF344 formula (Table 1) enhances its antihyperglycemic effects by targeting multiple pathways, thereby improving the pathological conditions associated with diabetes.

Moreover, piperine, which is the most abundant phytochemical compound of HF344 extract, has been reported to exhibit a beneficial role in DM mice by ameliorating pancreatic β-cell dysfunction [51], probably associated with the regulation of PI3K/Akt-mediated anti-apoptosis signaling. In addition, piperine demonstrates synergistic effects when combined with curcumin and metformin [52,53]. Piperine, in conjunction with the phytochemical components of HF344 extract predominantly comprising flavonoids, alkaloids, and terpenoids identified by UHPLC-DAD-MS/MS analysis as presented in Fig. 1, Table 3, and supplementary data, may contribute to the observed antihyperglycemic and antioxidant activities in this study through potential synergistic mechanisms [54]. These combined effects may hold promise for the management of diabetes and its complications, including foot ulcers [10,11].

Magnesium sulfate ($MgSO_4$) can help reduce blood glucose levels and improve glucose tolerance through several mechanisms [55–57]. Magnesium deficiency is associated with insulin resistance, and supplementation can improve insulin sensitivity in type 2 diabetic rats by stimulating GLUT4 gene expression, thereby lowering blood glucose levels [57]. Furthermore, $MgSO_4$ improved hyperglycemia by inhibiting FOXO1 gene and protein levels in the muscle and liver, and decreased blood glucose levels by suppressing phosphoenolpyruvate carboxykinase (PEPCK) enzyme activity in the gluconeogenesis pathway in the liver [58]. Moreover, a recent study reported that $MgSO_4$ could improve diabetic nephropathy [59], decrease oxidative damage by reducing ROS production, and play a critical role as an anti-inflammatory agent by inhibiting pro-inflammatory cytokines, such as NF-κB [56]. Therefore, $MgSO_4$ has been included in the present polyherbal formula to achieve a synergistic effect with other herbal components, particularly to reduce blood glucose levels and improve insulin resistance. In addition to its hypoglycemic effects, $MgSO_4$ also acts as a mild laxative, counteracts the astringent effects of tannins in other herbs, and contributes to bodily balance according to Thai traditional medicine principles [60].

The safety profile of HF344 extract was evaluated through acute oral toxicity testing according to the OECD425 toxicology test guideline, confirming its safety at doses up to 2000 mg/kg, exceeding the estimated 50 % lethal dose. This favorable safety profile may hold promise for the future clinical use of HF344 as a therapeutic polyherbal formula for T2DM.

Dose conversion from animal to human must be appropriately translated during new drug development and the animal dose should not be extrapolated to humans by a simple conversion method based only on body weight. Therefore, the present results can be extrapolated for clinical application in humans by employing the calculation of human equivalent dose (HED) suggested by the Food and Drugs Administration through the normalization of the body surface area and conversion factor depent on species indicated as "K_m" according to the formula shown in Equation (6) [61]:

$$\text{Human equivalent dose (HED)(mg / kg)} = \text{Animal dose } \left(\frac{mg}{kg}\right) \times \frac{Animal\ K_m}{Human\ K_m} \quad \text{(Eq. 6)}$$

where the animal dose used in this study was 500 and 1000 mg/kg, and K_m for mice and humans was 3 and 37, respectively [61].

Consequently, the HED was calculated using Eq. (6) and determined to be 40.5 and 81 mg/kg, which translates to a daily dose of 2432 and 4864 mg for a 60 kg human. Applying a safety factor of 10, as commonly practiced in clinical trials [61], the suggested initial doses, based on the animal doses of 500 and 1000 mg/kg, for the clinical trial, would be 243 and 485 mg/day/human.

Despite the promising outcomes of the HF344 formulation for future treatment of T2DM, several inherent limitations of this study warrant elucidation in subsequent investigations. Firstly, a histological examination of the pancreas in STZ-NA-induced DM mice should be conducted, since treatment with HF344 extract may induce histological alterations in pancreatic tissues, thereby substantiating observed improvements in insulin production. Another limitation pertains to the absence of direct evidence regarding HF344's ability to induce expression of the glucose transporter GLUT4 in physiological *in vivo* contexts. Considering GLUT4 role in facilitating cellular glucose uptake into muscle tissues, future investigations should integrate direct assessments of its expression and translocation *in vivo*. On the other hand, an emerging approach for modulating postprandial glycemic excursions involves inhibiting glucose absorption in the small intestine by targeting GLUT2 or sodium-dependent glucose transporter 1 (SGLT1). The lack of evidence regarding HF344's effects on GLUT2 and SGLT1 in this study further underscores its limitations. Hence, future investigations are warranted to explore its modulation of glucose uptake in the small intestine mediated by SGLT1 and GLUT2, to afford a more comprehensive understanding of the mechanisms underlying HF344 extract's actions in regulating postprandial hyperglycemia. Lastly, further research should focus on elucidating the long-term efficacy and safety of HF344 extract. It is recommended to conduct additional studies on chronic toxicity, particularly concerning potential hepatotoxicity, to explore its potential as a complementary or alternative therapy for individuals with T2DM in clinical settings.

5. Conclusions

The current study demonstrated the antihyperglycemic activity of the extract of HF344, a novel polyherbal formulation comprising fifteen herbal extracts along with magnesium sulfate. LC-MS analysis of HF344 extract revealed several phytoconstituents, with piperine identified as the major active compound. This activity is attributed to various mechanisms of antidiabetic actions, including stimulation of insulin secretion *in vitro*, glucose uptake in the sacs of the mouse small intestine *ex vivo*, upregulation of GLUT1 glucose transporters in insulin-responsive cells (specifically, L6 skeletal myoblasts cells), inhibition of glucose absorption via α-glucosidase inhibition, mitigation of insulin resistance *in vivo* (as evidenced by a reduction in HOMA-IR), and augmentation of insulin production and functionality of pancreatic β-cells *in vitro* (as demonstrated by recovery from STZ-induced apoptosis in RINm5F cells). Furthermore, acute oral toxicity testing has confirmed the safety profile of HF344 extract in mice. These findings underscore the efficacy and safety of the herbal formula in managing T2DM, thus warranting further exploration of the HF344 formulation in clinical settings.

Funding

This research was financially supported by Twin Lotus Co., Ltd., Bangkok, Thailand, and partly by Mahidol University.

Institutional Review Board Statement

The animal study protocol was approved by the Institutional Review Board of the Faculty of Pharmacy, Mahidol University (permission numbers: PYR 003/2019 and PYR003/2023).

Data availability statement

The data presented in the present study are available on request from the corresponding author on request.

CRediT authorship contribution statement

Worawan Kitphati: Writing – original draft, Project administration, Methodology, Data curation, Conceptualization. **Vilasinee Hirunpanich Sato:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Penchom Peungvicha:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Nakkawee Saengklub:** Writing – original draft, Methodology, Funding acquisition, Data curation. **Savita Chewchinda:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Sumet Kongkiatpaiboon:** Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation. **Arman Syah Goli:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Manaw Sangfuang:** Writing – original draft, Methodology, Investigation. **Jannarin Nontakham:** Writing – original draft, Methodology, Investigation, Formal analysis. **Nutputsorn Chatsumpun:** Writing – original draft, Methodology, Investigation. **Supachoke Mangmool:** Writing – original draft, Validation, Methodology, Investigation, Data curation. **Hitoshi Sato:** Writing – review & editing, Visualization, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to express sincere thanks to Mr. Mana Thabloha, Faculty of Pharmacy, for helping conduct the *in vivo* studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38703.

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