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Article

Insulinotropic and Beta-Cell Proliferative Effects of Unripe *Artocarpus heterophyllus* Extract Ameliorate Glucose Dysregulation in High-Fat-Fed Diet Induced Obese Mice

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Abstract: *Artocarpus heterophyllus*, familiar as jackfruit, is a tropical fruit highly valued not only for its nutritional content but also for its medicinal properties, including potential antidiabetic effects. In this study, ethanolic extract of unripe *Artocarpus heterophyllus* (EEAH) exhibited significant antidiabetic activity through multiple mechanisms. EEAH enhanced glucose-stimulated insulin secretion in BRIN-BD11 β -cells via K_{ATP} channel modulation and cAMP-mediated pathways, with partial dependence on extracellular calcium, and it also promoted β -cell proliferation. *In vitro* assays revealed its ability to inhibit starch digestion and glucose diffusion, indicating delayed carbohydrate digestion and absorption. In high-fat-fed (HFF) obese mice, Acute and chronic oral administration of EEAH improved oral glucose tolerance, reduced fasting blood glucose, decreased body weight, and normalized food and fluid intake. Lipid profile analysis showed increased HDL and reduced total cholesterol, LDL, and triglycerides, while higher doses of EEAH also enhanced gut motility. Phytochemical screening revealed the presence of bioactive compounds such as alkaloids, tannins, flavonoids, saponins, steroids and, terpenoids, which are likely responsible for these therapeutic effects. These findings highlight EEAH as a promising natural candidate for adjunctive therapy in managing type 2 diabetes and associated metabolic disorders and emphasize the importance of future multi-omics studies to elucidate its molecular targets and pathways.

Keywords: insulin resistance; hyperglycaemia; type 2 diabetes mellitus; beta-cell proliferation; lipids; *Artocarpus heterophyllus*

1. Introduction

Diabetes mellitus (DM), is a complex multifactorial metabolic disorder, characterized by excessive glucose in the circulation resulting from an impaired insulin sensitivity, deficient secretion of the anabolic hormone insulin, or both [1,2]. Globally, DM presents a significant public health challenge; the International Diabetes Federation (IDF) estimated that approximately 537 million people had DM in 2021, and this number is projected to rise to 783 million by 2045 [3]. Nearly, 10% of adults worldwide are diagnosed with diabetes mellitus (DM), being a major contributor to increased morbidity and mortality [4].

The development and progression of diabetes fundamentally stem from inadequate insulin production or impaired insulin action, both of which are critical for maintaining metabolic balance. Insulin is an essential peptide hormone produced by β -cells, plays a crucial role in regulating metabolic processes [5,6]. Its synthesis begins with the transcription of the insulin gene into mRNA, followed by translation into pre-proinsulin. This precursor is subsequently processed, first into proinsulin and then further into mature insulin and C-peptide. [6,7]. Insulin promotes glucose uptake in skeletal muscle by facilitating its absorption and conversion to glycogen, and it also stimulates glycogen storage and inhibits excessive glucose production in the liver [8].

Diabetes presents with diverse phenotypes. Type 1 diabetes (T1D) is an autoimmune disorder marked by the destruction of pancreatic β -cells, causing absolute insulin deficiency and dysregulated α -cell activity that leads to excessive glucagon secretion. [1,2,9,10]. In contrast, type 2 diabetes mellitus (T2DM), accounts for 90% of diabetes cases, is a heterogeneous condition primarily driven by impaired insulin secretion from pancreatic β -cells and reduced insulin function due to insulin resistance [11,12]. Acute complications of diabetes include life-threatening conditions such as diabetic ketoacidosis (DKA) and severe hypoglycemia. Chronic hyperglycemia leads to both microvascular damage (retinopathy, nephropathy, and neuropathy) and macrovascular complication, thereby increasing the risk of diabetic foot ulcers and premature mortality [13].

Research indicates that obesity is a major risk factor for the development of type 2 diabetes mellitus (T2DM), with approximately 90% of individuals with the disease being overweight or obese [14]. While diet and exercise aid weight loss in T2DM, they often fall short in sustaining optimal glycemic control [15]. Therefore, a range of antidiabetic medications, including metformin, sulfonylureas, meglitinides, thiazolidinediones, SGLT2 inhibitors, GLP-1 mimetics, and DPP-IV inhibitors, are essential for effective blood glucose management. However, these medications are often unaffordable and inaccessible for individuals in underprivileged communities and can also have undesirable side effects [16]. Consequently, affordable and effective alternatives are needed to mitigate the detrimental effects of uncontrolled diabetes [15,17].

Traditionally, people worldwide have relied on various herbs and medicinal plants to manage and treat diabetes mellitus [15,17]. *Artocarpus heterophyllus*, generally known as jackfruit, which belongs to member of the Moraceae family and is cultivated in tropical regions particularly in countries such as India, Bangladesh, Sri Lanka, Thailand, Indonesia, the Philippines, Brazil, and parts of East and West Africa [18–24]. It has been utilized by indigenous people for centuries to treat diverse illnesses including diabetes, inflammation, infection, cancer, and CVD due to its innumerable medicinal properties [19,20]. *A. heterophyllus* is reported to be abundant in dietary fiber, carbohydrates, minerals, carboxylic acids and vitamins such as thiamine and ascorbic acid [18,21]. Previous studies have shown that *A. heterophyllus* possesses antioxidant, anti-inflammatory, anti-fungal, anti-diabetic, anti-bacterial, immunomodulatory and wound healing properties [20,22]. Recent studies suggested that *A. heterophyllus* lowers blood glucose, improves oral glucose tolerance, and body weight [23–25]. Based on these studies, our present aim was to investigate the insulintropic and antidiabetic properties of *A. heterophyllus*, as well as the underlying mechanisms of action on pancreatic β -cells, using both *in vitro* and *in vivo* models.

2. Materials and Methods

2.1. Preparation and Extraction of EEAH

Green *Artocarpus heterophyllus* (unripe jackfruit) was acquired from Jahangirnagar University, Savar, Dhaka, Bangladesh and botanical accession number 87,070 was endorsed by botanical taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka. The unripe jackfruit was meticulously cleaned, allowed to air dry for several hours and afterwards dried further in an oven at 45°-50° before emanating to ethanolic extract preparation. The dried components were further processed and subsequently ground into a fine powder using a grinding machine. Two hundred grams of the powder were then added to 1 liter of 80% (v/v) ethanol and placed on an orbital shaker

at 900 rpm for 48-72 h. The resulting mixture was then filtered using Whatman No. 1 filter paper, and the filtrate was dried under vacuum using a rotary evaporator. The final extracted semi-solid, gummy crude extract of *A. Heterophyllus* was stored at 4°C until experimental use [26].

2.2. Studies of Insulin Release In Vitro

Clonal pancreatic BRIN-BD11 β -cells were used to analyze the impact of EEAH on insulin release *in vitro* [27]. With multiple varying concentrations (8–5000 $\mu\text{g/ml}$) of EEAH, BRIN-BD11 β -cells were incubated at 37°C for 20 min at 5.6 mM glucose [28]. The supernatants were collected and stored at -20 °C for insulin radioimmunoassay [29]. To determine the secretory pathways activated by EEAH, its effects were evaluated in the presence of various insulin secretagogues and inhibitors. These included tolbutamide (a sulfonylurea and K_{ATP} channel blocker), IBMX (a phosphodiesterase inhibitor), 30 mM KCl, 10 mM alanine, diazoxide (a K_{ATP} channel opener), and verapamil (a voltage-dependent Ca^{2+} channel blocker). Both KCl and alanine induce plasma membrane depolarization and Ca^{2+} influx, with alanine primarily achieving this through Na^{+} co-transport and ATP-generating metabolism. [17,30].

2.3. β -Cell Proliferation In Vitro

To investigate the effects of ethanol extract of *A. heterophyllus* (EEAH) on β -cell proliferation, BRIN-BD11 cells were treated with EEAH at concentrations of 40 and 200 $\mu\text{g/mL}$, and glucagon-like peptide-1 (GLP-1) (10^{-6} M), as a positive control. Ki-67 immunostaining was used to quantify cell proliferation. Briefly, cells were seeded onto coverslips (40,000 cells/coverslip) and incubated with EEAH or GLP-1 for 18 h at 37°C. After washing with phosphate-buffered saline (PBS), cells were fixed with 4% paraformaldehyde. Antigen retrieval was performed using citrate buffer (90°C, 20 min), and non-specific binding was blocked with 1.1% bovine serum albumin (BSA) for 30 min. Cells were then incubated with a Ki-67 primary antibody (1:500; Abcam, ab15580), followed by an Alexa Fluor® 488 secondary antibody (1:400, Invitrogen, A-11008). Coverslips were washed with PBS, mounted onto slides, and visualized using an Olympus System fluorescence microscope equipped with a DP70 camera adapter. Proliferation was quantified as the percentage of Ki-67-positive cells relative to the total number of cells analyzed [31].

2.4. In Vitro Starch Digestion

The effect of EEAH on starch digestion was examined by mixing 100 mg/50ml starch solution (Sigma-Aldrich, St. Louis, MO, USA) with/without EEAH, and acarbose. Following dilution, the mixtures were incubated with 0.01% heat stable α -amylase from *Bacillus leicheniformis* and 0.1% amyloglucosidase from *Rhizopus sp.* (Sigma-Aldrich, St. Louis, MO, USA) for 20 and 30 min at 80°C and 60°C respectively. Afterwards, the samples were collected and stored for analysing the concentration of glucose using liquid Glucose Oxidase-phenol 4 aminophenazone (GOD-PAP) method (Randox GL 2623) [28,29]. Alpha glucosidase inhibitor, Acarbose was used as a reference drug.

2.5. In Vitro Glucose Diffusion

The implications of EEAH on glucose diffusion *in vitro* were examined utilizing a cellulose ester dialysis tube (CEDT) (20 cm x 7.5 mm, Spectra/Por®CE layer, MWCO: 2000, Spectrum, Amsterdam, The Netherlands). In a dialysis tube, 2 mL of 0.9% NaCl was added along with 220 mM glucose in the presence or absence of EEAH. The tubes were tightly sealed and then placed into 50 mL centrifuge tubes (Orange Scientific, Orange, CA, USA) filled with 45 mL of 0.9% NaCl. Subsequently, the tubes were agitated on an orbital shaker at 37°C for 24 hr. Following incubation, the samples were collected. The amounts of glucose diffused into the external solution was measured using GOD/PAP method (Randox GL 2623) [32,33].

2.6. Animals

Male Swiss albino mice, approximately two weeks old and weighing 20-25g, were obtained from the Animal Resources Facility, ICDDR, Dhaka, Bangladesh. They were housed at a controlled temperature of 22±3°C and humidity of 55-65%, with *ad libitum* access to food and water, under an automated 12-h light/dark cycle in the Animal House at Independent University, Bangladesh. Following a one-week acclimation period, the mice were divided into two groups based on body weight: a normal control group (NC) fed a standard diet (10% fat, 30% protein, and 60% carbohydrate) and a high-fat-fed (HFF) group fed a high-fat-fed diet (19.4% powdered normal animal food, 17.5% sugar, 21% beef fat, 39.5% condense milk, 1.1% Vitamin-B complex and 1.5% salt) for 6 to 8 weeks. For subsequent experiments, the HFF group was further subdivided into four groups: a high-fat-fed diet control group (HFF) (Group 2), two treatment groups (Group 3 and 4), and a standard treatment group (Group 5). HFF diet-induced obese type 2 diabetic mice were characterized by fasting blood glucose levels exceeding the normal range (>6.0 mmol/L) and a body weight of 40-45g. The normal control and high-fat-fed diet control groups received saline (5 mL/kg). The treatment groups received 250 and 500 mg/5mL/kg of EEAH, respectively, and the standard treatment group received glibenclamide (5 mg/5mL/kg) twice daily (BID) for 60 days. Each group contained n=6 mice. This study was approved by the Institutional Review Board (IRB) at Independent University, Bangladesh (IUB) on September 1, 2022 for animal experiments. The experiments were conducted in accordance with the animal Welfare Act 2019 of Bangladesh, UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63/EU. Experiments were performed under UK Home Office Animal license PIL1822, ensuring ethical animal care and minimizing distress. The groups were divided as follows:

Group 1: Lean control (saline)

Group 2: High fat fed diet control (saline)

Group 3: High fat diet + EEAH (250 mg/5 mL/kg)

Group 4: High fat diet + EEAH (500 mg/5 mL/kg)

Group 5: High fat diet + Glibenclamide (5 mg/5 mL/kg)

2.7. Acute and Chronic Oral Glucose Tolerance Test

Oral glucose tolerance tests (OGTT) were performed on high-fat-fed (HFF) diet-induced obese mice at days 0, 15, 30, 45, and 60 of the 60-day study to monitor glucose handling over time. The OGTT conducted on day 0 was carried out prior to the initiation of treatment to assess baseline glucose tolerance, while subsequent tests evaluated the progression and potential therapeutic effects of the test agents. HFF diet mice were starved for 12h prior to the experiment. Blood glucose were measured using tail vein bleeding at particular time intervals prior to (0 min) and after (30, 60, 120 & 180-min) oral gavage of glucose (2.5 gm/5 mL/kg, body weight, control) with/without EEAH (250 and 500 mg/5 mL/kg, body weight) or STD, glibenclamide (5 mg/5 mL/kg, body weight). Blood glucose levels were determined using an Ascencia Contour glucose meter and glibenclamide, an insulin secretagogue, was used as the reference drug [12].

2.8. Chronic Study of Blood Glucose, Body Weight, Food and Fluid Intake

A 60-day long-term study was conducted on high-fat-diet-induced obese mice. Various parameters, including fasting blood glucose, body weight, food and fluid intake, were measured every three days. All groups, including the high-fat-diet-induced obese mice and the normal control group, were fasted for 12 h prior to these measurements. The treatment groups received EEAH at doses of 250 and 500 mg/5 mL/kg, respectively. The positive control group received glibenclamide (5 mg/5 mL/kg), while both the high-fat-diet control group and the normal control group received saline

(5 mL/kg). An Ascensia Contour Blood Glucose Meter (Bayer, Newbury, UK) was used to measure blood glucose levels [12,30,34].

2.9. Lipid Profile Test

Throughout the 60-day study, high-fat-diet-induced obese mice received twice-daily oral treatments of either EEAH (250 and 500 mg/5 mL/kg) or glibenclamide (5 mg/ 5 mL/kg). A lipid profile test was conducted as previously described [15]. At the end of study, the mice were euthanized, and blood was collected from the heart using 5 mL syringes into heparinized microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) to prevent coagulation. The blood samples were centrifuged at 12,000 rpm for 5 min, and the resulting plasma serum was separated. High-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), and total cholesterol (TC) levels were then analysed using COD-PAP, GPO-PAP (Elabsience Biotechnology Co., Ltd., TX, USA), and CHOD-PAP (Biolabo SAS, Maizy, France) reagents with an automated analyzer [35,36].

2.10. Gut Motility In Vivo

At the end of the 60-day chronic study, mice were sacrificed and gastrointestinal motility was assessed by measuring the distance traveled by a barium sulfate (BaSO_4) milk solution in mice fasted for 20 h. One hour before administration of the BaSO_4 solution, the treatment groups received EEAH (250 and 500 mg/5 mL/kg), bisacodyl (10 mg/5 mL/kg), or loperamide (5 mg/5 mL/kg). The high-fat-fed (HFF) control and the normal control groups received only the BaSO_4 solution. Fifteen minutes after administration of the BaSO_4 milk solution, the mice were euthanized, and their entire small intestines were extracted. The BaSO_4 transit distance was then measured and expressed as a percentage of the total small intestine length (from the pylorus to the ileocecal junction) [37,38].

2.11. Phytochemical Screening

To ascertain the source of alkaloids, tannins, flavonoids, saponins, steroids, terpenoids, glycosides and reducing sugar, a phytochemical analysis of EEAH was performed. In order to determine the presence of alkaloids, 2 mL of EEAH was acidified with hydrochloric acid (HCl), followed by 1 mL of Dragendroff's solution. The emergence of a red colour implies the presence of alkaloids. To determine the presence of tannins, 2 mL of EEAH was mixed with 10% lead acetate. A white precipitate formation upon mixing, confirming the presence of tannins. The presence of flavonoids was determined by heating a mixture of 1.5 mL of methanol and 4 mL of EEAH, followed by the addition of metal magnesium and a few drops of HCl to the heated mixture, which resulted in the development of pink colour. To evaluate the presence of saponins, 1 mL of EEAH was added to 9 mL of distilled water, resulting in the formation of a stable foam. The presence of steroids was analysed by adding 2 mL of EEAH with 10 mL of chloroform, followed by 1 mL of acetic anhydride, and 2 mL of sulphuric acid, leading in the formation of bluish-green colour. To test for terpenoids, 1g of EEAH was dissolved in 2mL of chloroform. Then, 3mL of strong sulfuric acid was carefully added to form a distinct layer. A reddish-brown color at the interface indicated the presence of terpenoids. The presence of glycoside was detected by mixing 1 mL of EEAH with a few drops of glacial acetic acid, ferric chloride, followed by concentrated sulfuric acid, which resulted in a bluish-green colour. In order to identify the presence of reducing sugars, 1 mL of EEAH, 1 mL of water and a few drops of Fehling's reagent were heated, resulting in the formation of red-brick colour. [32,39,40].

2.12. Statistical Analysis

GraphPad Prism version 10 was used for statistical analysis. Data were assessed using a two-tailed unpaired Student's t-test or one-way or two-way analysis of variance (ANOVA) with a Bonferroni post hoc test. Results were presented as mean $\pm$ SEM, with statistical significance defined as $p < 0.05$.

3. Results

3.1. Insulin Release with EEAH

At 5.6 mM glucose, EEAH significantly stimulated insulin secretion from BRIN-BD11 β -cells in a concentration-dependent manner across a range of 40–5000 $\mu\text{g/mL}$ ($p < 0.05$ to $p < 0.001$; Figure 1A). Alanine (10 mM), served as a positive control, remarkably increased insulin secretion ($p < 0.001$; Figure 1A).

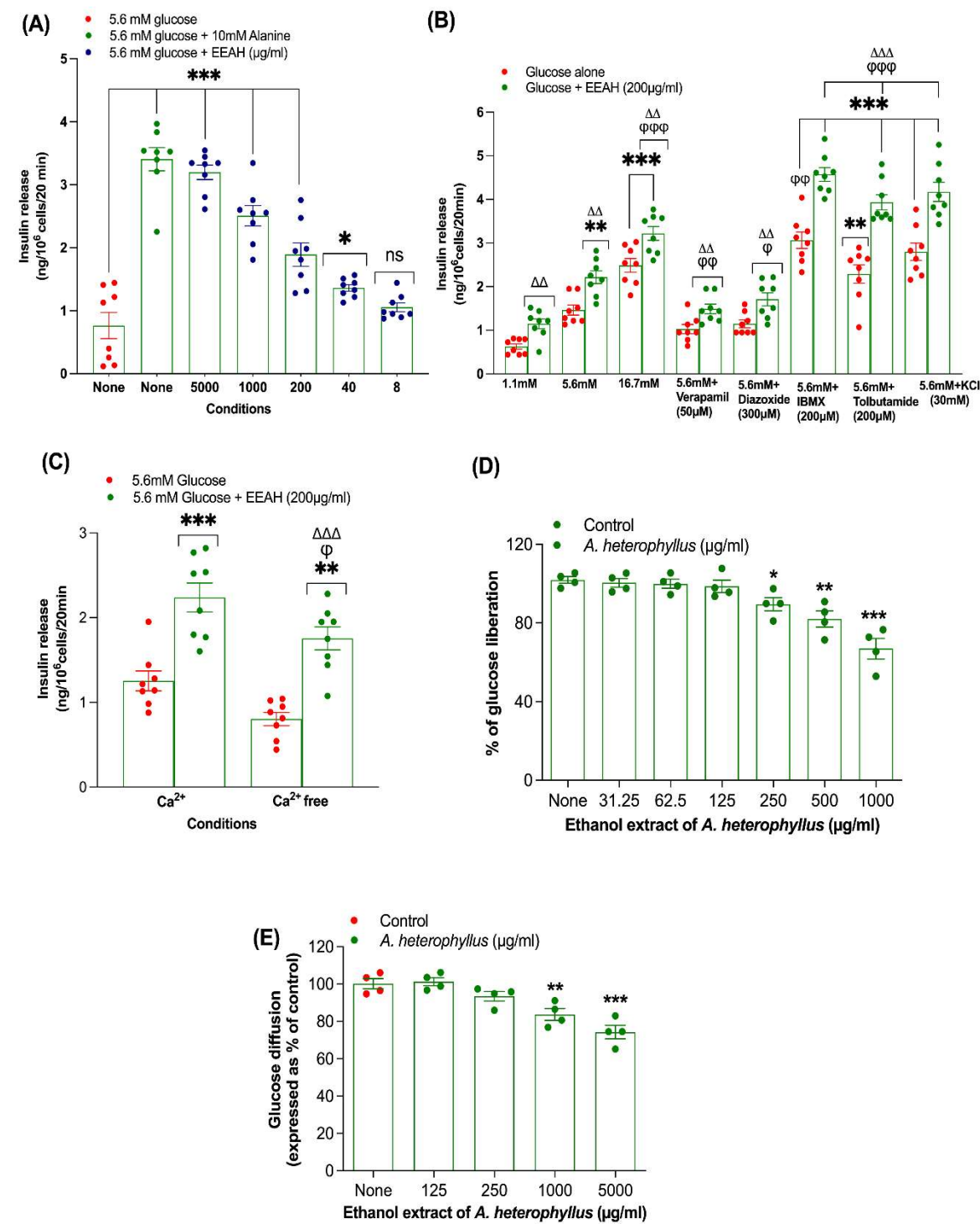


Figure 1. Effects of Ethanol extract of unripe *A. heterophyllum* (EEAH) on insulin secretion from (A) clonal pancreatic BRIN BD11 β -cells alone, (B) with known modulators or inhibitors, (C) with or without extracellular

calcium, (D) starch digestion, and (E) glucose diffusion. Percentage of glucose liberation from starch digestion and glucose diffusion *in vitro* were represented in the scatter dot plot. Values are mean $\pm$ SEM; $n = 8$ for insulin secretion and $n = 4$ for starch digestion and glucose diffusion. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control. $\phi p < 0.05$ and $\phi\phi\phi p < 0.001$ compared to 5.6 mM glucose with EEAH. $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$ compared to respective incubation without EEAH. EEAH, ethanol extract of *A. heterophyllus* (unripe fruit).

3.2. Insulin Release with EEAH, Known Modulators and Absence of Extracellular Ca^{2+}

To elucidate the insulinotropic mechanisms of non-cytotoxic concentrations (200 $\mu\text{g/mL}$) of EEAH, further investigations were conducted (Figure 1B). As depicted in Figure 1B, EEAH-stimulated insulin secretion exhibited potentiation in the presence of key modulators: high glucose (16.7 mM, $p < 0.01$), the non-selective phosphodiesterase inhibitor IBMX ($p < 0.001$), and the sulfonylurea receptor 1 (SUR1) agonist tolbutamide ($p < 0.001$). The synergistic action of IBMX, leading to elevated intracellular cyclic AMP, and tolbutamide, promoting SUR1 closure and subsequent membrane depolarization, amplified both cAMP production and insulin secretion. Conversely, the K_{ATP} channel opener diazoxide and the L-type voltage-gated calcium channel (VGCC) antagonist verapamil attenuated EEAH-induced insulin release ($p < 0.05$ - 0.01 ; Figure 1B), as evidenced by reduced insulin secretion when incubated with Ca^{2+} free buffer ($P < 0.05$; Figure 1C). Notably, EEAH retained its capacity to stimulate insulin secretion from cells depolarized with 30 mM KCl ($p < 0.001$; Figure 1B), indicating a mechanism downstream of membrane potential. However, the absence of extracellular Ca^{2+} diminished, though did not completely abrogate, EEAH's insulin-releasing activity ($p < 0.001$; Figure 1C), underscoring the partial dependence on extracellular calcium for its full insulinotropic effect.

3.3. Proliferation In Vitro with EEAH

Figure 2A-D displays BRIN BD11 β -cell proliferation images for three conditions: (A) Control, (B) GLP-1 (10^{-6} M), and (C,D) EEAH (at concentrations of 40 and 200 $\mu\text{g/mL}$). Quantitative analysis (Figure 2E) revealed that EEAH significantly enhanced BRIN-BD11 β -cell proliferation at both 40 $\mu\text{g/mL}$ ($p < 0.01$) and 200 $\mu\text{g/mL}$ ($p < 0.001$) compared to the control. The positive control, GLP-1 (10^{-6} M), demonstrated the most potent effect on BRIN-BD11 β -cell growth ($p < 0.001$; Figure 2E).

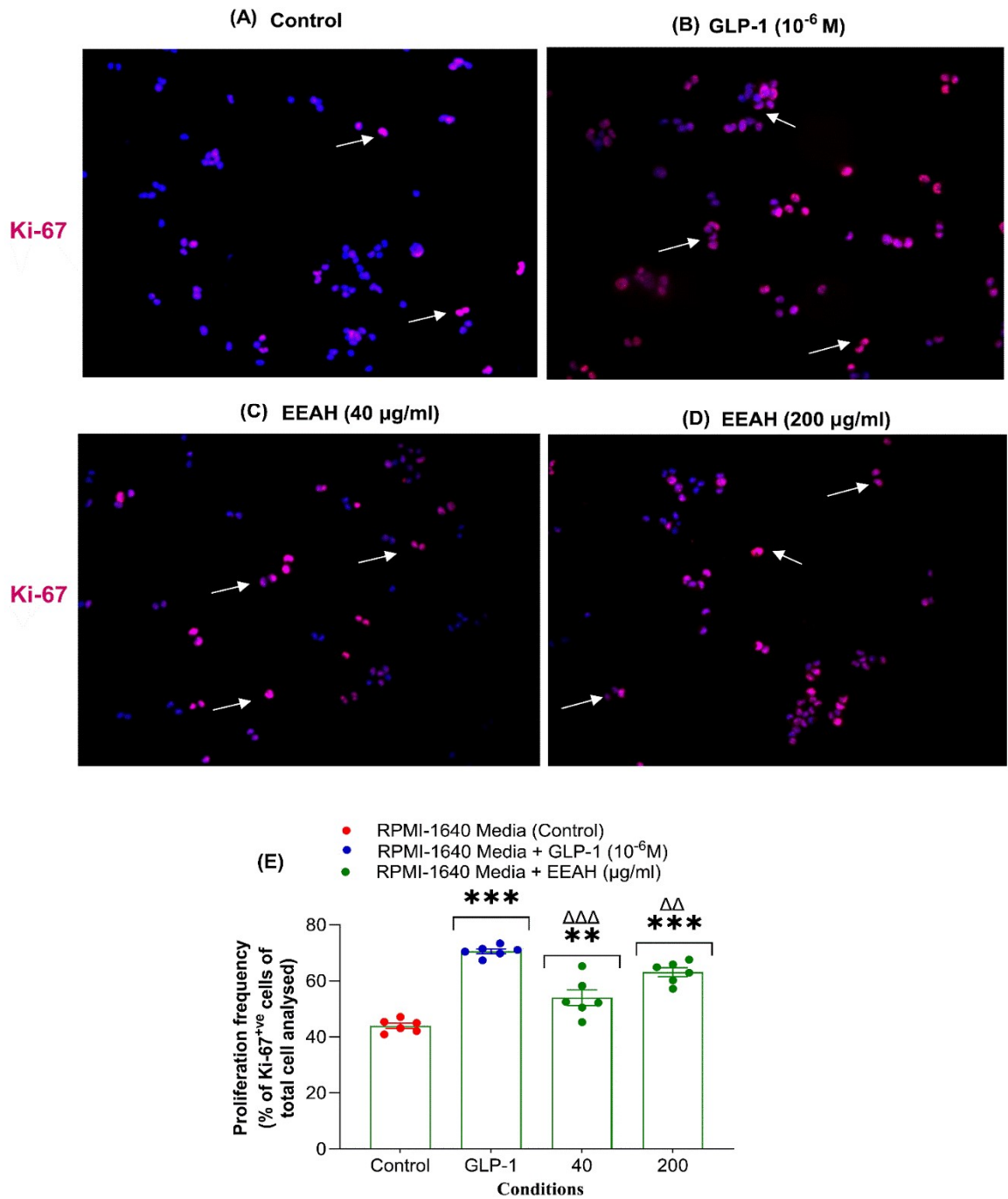


Figure 2. Effects of Ethanol extract of unripe *A. heterophyllus* (EEAH) on (A-E) BRIN-BD11 β-cells proliferation. BRIN-BD11 cells were cultured (18 h) with EEAH (40 and 200 µg/ml) or GLP-1 (10⁻⁶ M) and proliferation assessed by Ki-67 staining. Representative images depict cells stained with DAPI (blue) and Ki-67 (red), with arrows indicating Ki-67-positive (proliferating) cells. Figure 2A-D shows BRIN BD11 β-cell proliferation images under four conditions: (A) Control, (B) GLP-1 (10⁻⁶ M), (C) EEAH at 40 µg/ml, and (D) EEAH at 200 µg/ml. Percentage of Ki-67⁺ve cells were represented in the scatter dot plot. All values are mean ± SEM with n = 6. **, *** *p* < 0.01-0.001 compared with control. ΔΔ, ΔΔΔ *p* < 0.01-0.001 compared with GLP-1 (10⁻⁶ M).

3.4. Starch Digestion In Vitro with EEAH

Figure 1D illustrates the impact of Acarbose and EEAH on starch digestion. The positive control, Acarbose (62.5–1000 µg/mL), demonstrated a concentration-dependent reduction in starch digestion

ranging from 20% to 80% (Data not shown). EEAH also significantly inhibited starch digestion (11–33%, $p < 0.05$ –0.001, Figure 1D) at higher concentrations (250–1000 $\mu\text{g/mL}$).

3.5. Glucose Diffusion In Vitro with EEAH

EEAH significantly ($p < 0.01$ –0.001; Figure 1E) inhibited glucose diffusion over a 24 h incubation period compared to the control. As depicted in Figure 1E, the extract reduced total glucose diffusion by 16–26%, with the highest inhibition (26%) observed at a concentration of 5000 $\mu\text{g/mL}$.

3.6. Oral Glucose Tolerance Test with EEAH

Following oral gavage of EEAH (250 and 500 mg/5 mL/kg) in conjunction with glucose (2.5 gm/5 mL/kg), significantly ($p < 0.05$ –0.001) improved oral glucose tolerance at 30, 60, and 120 min on 0, 15, 30, and 60 days of studies (Figure 3A–D) in HFF diet induced obese mice as compared to high-fat-fed diet induced obese control mice. Treatment with EEAH at doses of 250 and 500 mg/5 mL/kg improved oral glucose tolerance by 1.2-fold and 1.4-fold at 30 and 60 min, respectively, consistently throughout the studies. In comparison to the HFF diet induced obese control mice, the standard drug glibenclamide (5 mg/5 mL/kg) also significantly ($p < 0.05$ –0.001) decreased blood glucose levels at 30, 60, and 120 min on days 0, 15, 30, and 60 (Figure 3A–D).

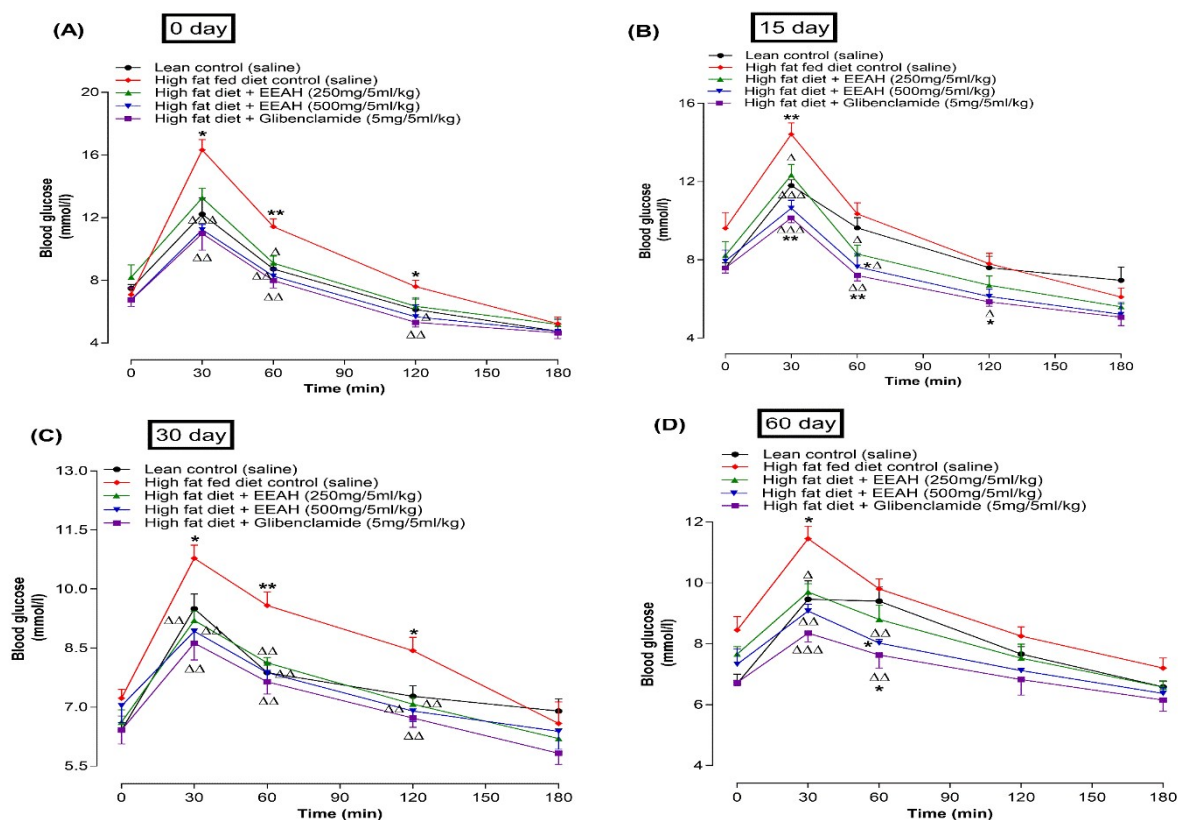


Figure 3. Acute and chronic effects of ethanol extract of unripe *A. heterophyllum* (EEAH) in high-fat-fed mice on oral glucose tolerance at (A) 0, (B) 15, (C) 30 and (D) 60 days respectively expressed as line graphs. High-fat-fed mice fasted for overnight were used to measure the blood glucose from the tail tips prior to and after receiving glucose (2.5 gm/5 ml/kg, body weight, control) with or without EEAH (250 and 500 mg/5 ml/kg, body weight) or glibenclamide (5 mg/5 ml/kg). Values are the mean $\pm$ SEM for $n = 6$ mice. *, ** $p < 0.05$ –0.01 compared to lean control (saline), Δ , $\Delta\Delta$, $\Delta\Delta\Delta$ $p < 0.05$ –0.001 compared to high-fat-fed diet control mice. Glibenclamide was used as a positive control.

3.7. Chronic Study of Fasting Blood Glucose, Body Weight, Food and Fluid Intake with EEAH

Oral administration of EEAH (250 and 500 mg/5 mL/kg) and glibenclamide (5 mg/5 mL/kg) twice-daily showed remarkable reductions in fasting blood glucose levels in HFF diet induced obese mice during the study period of 60 days (Figure 4A). In comparison to HFF alone, EEAH at doses of 250 and 500 mg/5 mL/kg considerably ($p < 0.05-0.001$) lowered blood glucose levels from day 27 and onwards, with 500 mg/5 mL/kg dose showing greater potency than 250 mg/5 mL/kg dose. Additionally, the standard drug glibenclamide (5 mg/5 mL/kg) consistently decreased ($p < 0.05-0.001$; Figure 4A) blood glucose throughout the study period.

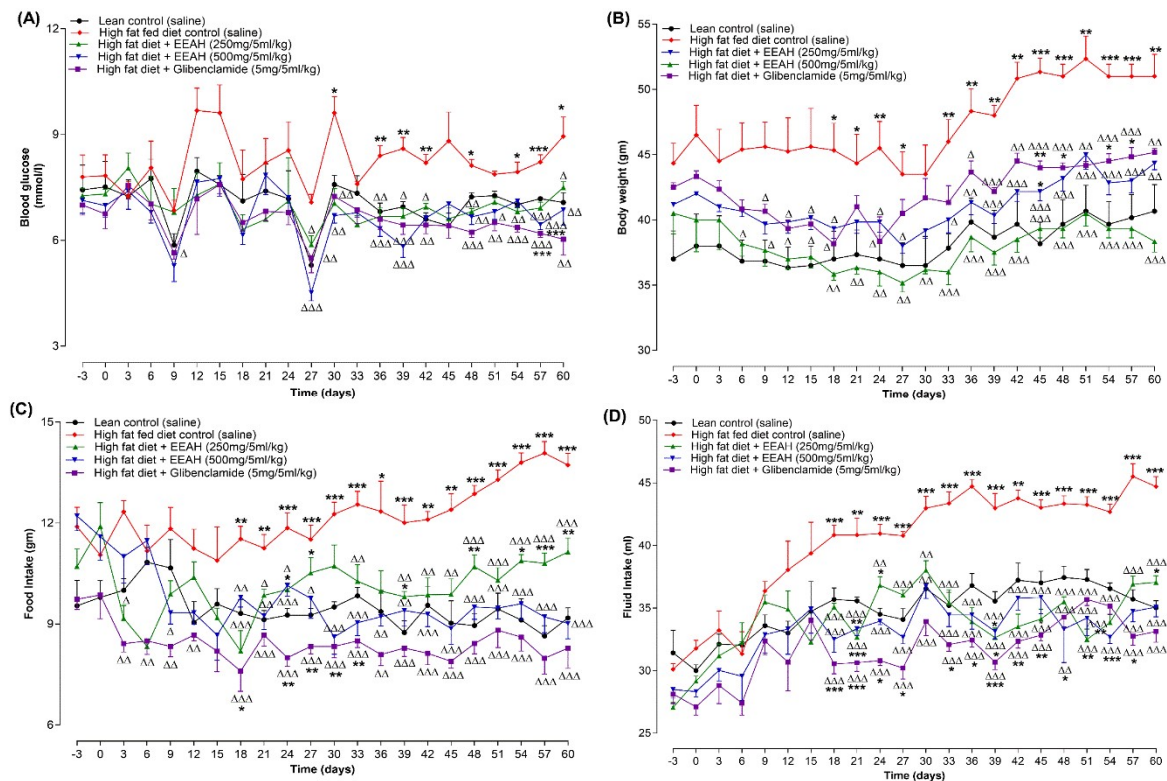


Figure 4. Effects of 60-days chronic treatment (twice-daily) with ethanol extract of unripe *A. heterophyllus* (EEAH) on (A) body weight, (B) fasting blood glucose, (C) food intake, (D) fluid intake in high-fat-fed mice represented as line graphs. Parameters were measured at 3-day intervals prior to and after oral administration of either EEAH (250 and 500 mg/5 mL/kg, body weight) or glibenclamide (5 mg/5 mL/kg, body weight) twice daily. Values are the mean $\pm$ SEM for $n = 6$ mice. *, **, *** $p < 0.05-0.001$ compared to control (saline). Δ, ΔΔ, ΔΔΔ $p < 0.05-0.001$ compared to high-fat-fed diet control mice. As a positive control, glibenclamide was used.

Oral gavage of EEAH (250 and 500 mg/5 mL/kg) and glibenclamide (5 mg/5 mL/kg) twice daily improved body weight in HFF mice during 60-day study period (Figure 4B). Compared to the HFF group alone, EEAH at doses of 250 and 500 mg/5 mL/kg gradually ($p < 0.05-0.001$) decreased body weight from day 6 and onwards. However, 500 mg/5 mL/kg dose showed a more consistent effect from day 18 and onwards. In addition, glibenclamide (5 mg/5 mL/kg) showed a remarkable ($p < 0.05-0.001$; Figure 4B) improvement in the body weight of HFF diet-induced obese mice from day 36 and onwards.

Oral administration of EEAH (250 and 500 mg/5 mL/kg) twice daily for 60 days significantly ($p < 0.05-0.001$; Figure 4C,D) improved food and fluid intake consistently from day 18 compared to HFF diet-induced obese control mice. Similarly, glibenclamide (5 mg/5 mL/kg) noticeably ($p < 0.01-0.001$; Figure 4C, D) decreased food and fluid intake consistently throughout the studies.

3.8. Lipid Profiling with EEAH

Long-term treatment (twice daily for 60 days) with EEAH (250 and 500 mg/5 mL/kg) or glibenclamide (5 mg/5 mL/kg) effectively ($p < 0.05$ - 0.01) elevated HDL cholesterol, while significantly ($p < 0.05$ - 0.001 ; Figure 5A-D) reduced total cholesterol, LDL and triglyceride levels, respectively, in high-fat-fed diet induced obese mice. High dose of EEAH at 500 mg/5 mL/kg showed the most potent antihyperglycemic effect. A sulfonylurea drug, glibenclamide (5 mg/5 mL/kg), also increased ($p < 0.05$, Figure 5A) HDL concentrations and reduced ($p < 0.05$ - 0.01 ; Figure 5B-D) total cholesterol, LDL as well as triglyceride levels compared to high-fat-fed diet induced obese mice alone.

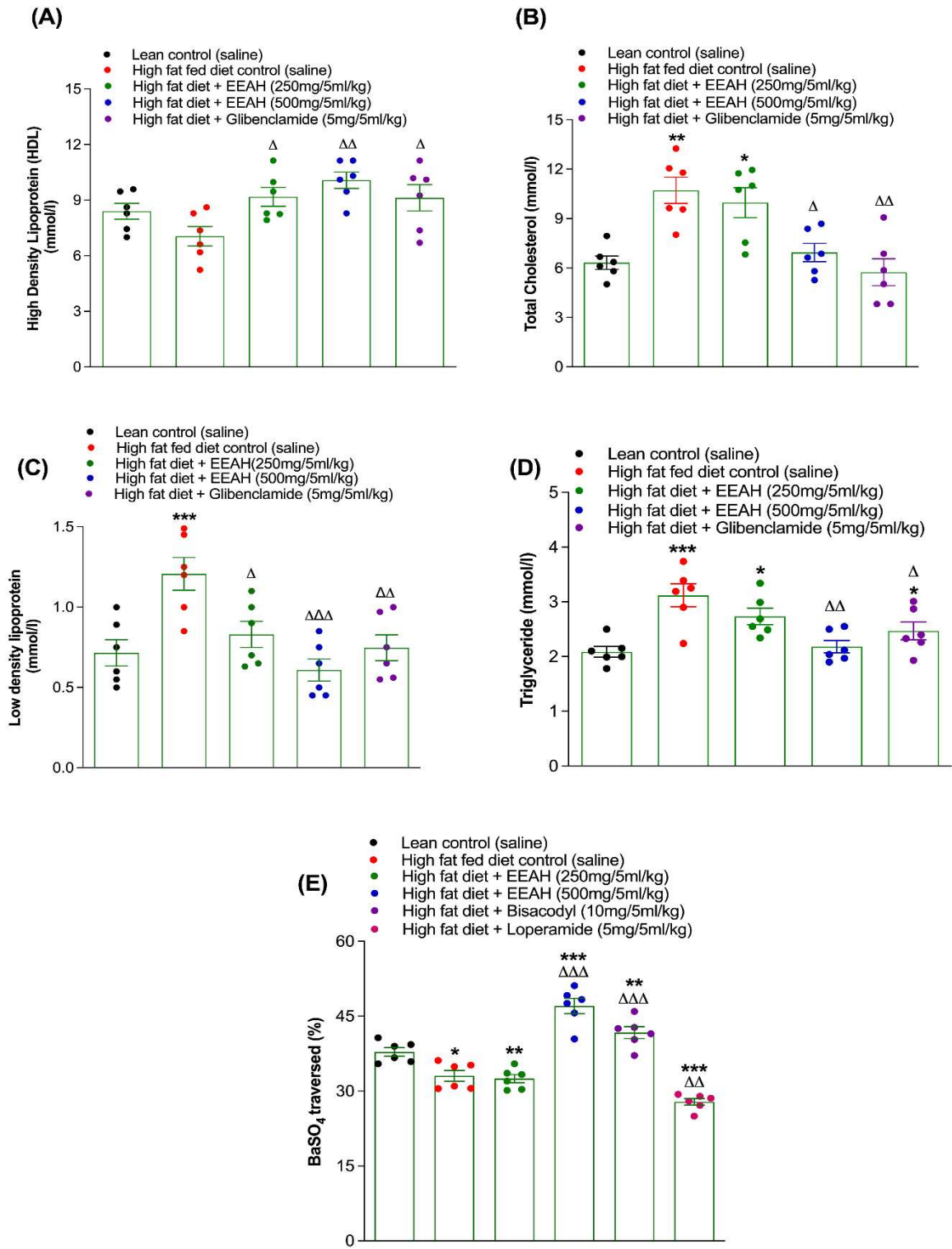


Figure 5. Effects of 60-days (twice-daily) oral administration of ethanol extract of unripe *A. heterophyllum* (EEAH) on (A) HDL cholesterol, (B) total cholesterol, (C) triglyceride and (D) LDL cholesterol and (E) gut motility in lean mice and high-fat-fed mice expressed as scatter dot plot. Parameters were measured on day 60 following twice-daily treatment with/without EEAH (250 and 500 mg/5 ml/kg, body weight) or glibenclamide (5 mg/5 ml/kg, body weight) or loperamide (5 mg/5 ml/kg) or bisacodyl (10 mg/5 ml/kg). Gastrointestinal motility was assessed by measuring BaSO₄ transit length in 20h fasted mice after ingestion of BaSO₄ solution. Values are expressed as mean ± SEM with n = 6 mice. *, **, *** $p < 0.05$ -0.001 compared to control (saline) and Δ , $\Delta\Delta$ $p < 0.05$ -0.01 compared to high-fat-fed diet control mice. As a positive control, glibenclamide, Loperamide and bisacodyl were used.

3.9. Gut Motility with EEAH

Following 60 days of treatment with EEAH at 250 mg/5 mL/kg, no significant changes was observed in gastrointestinal motility (Figure 5E). However, EEAH at 500 mg/5 mL/kg significantly ($p < 0.001$; Figure 4E) ameliorated gut motility. Additionally, treatment with the stimulant laxative bisacodyl (10 mg/5 mL/kg) and the sulfonylurea drug glibenclamide considerably ($p < 0.001$ and $p < 0.05$; Figure 4E) stimulated gut motility, whereas the antidiarrheal agent loperamide (5 mg/5 mL/kg) was associated with decrease ($p < 0.01$, Figure 4E) in intestinal motility.

3.10. Phytochemical Screening with EEAH

To identify potential anti-diabetic phytochemicals, present in unripe *A. heterophyllum* ethanolic extract, additional phytochemical screening was performed. The presence of alkaloids, tannins, flavonoids, saponins, terpenoids and steroids has been identified in EEAH (Table 1).

Table 1. Phytochemical screening of ethanol extract of *A. heterophyllum* (EEAH) unripe fruit.

Group Test	Observation
Alkaloids	+
Tannins	+
Flavonoids	+
Saponins	+
Steroids	+
Terpenoids	+
Glycoside	–
Reducing Sugar	–

‘+’ indicates present; ‘-’ indicates absent.

4. Discussion

Medicinal plants exhibit natural antioxidant properties which serve as an effective source of herbal glucose lowering medicine, particularly due to their bioactive phytochemicals such as flavonoids, saponins, tannins, phenolics, and alkaloids. These compounds can help in enhancing endocrine pancreatic function by the stimulation of insulin secretion or modulating intestinal glucose absorption [41]. This study investigated the antidiabetic potential of unripe *Artocarpus heterophyllum* (EEAH) extract via comprehensive *in vitro* and *in vivo* analyses to elucidate its mechanisms in regulation of blood glucose homeostasis.

The ethanolic extract of EEAH demonstrated a significant insulinotropic effect by enhancing insulin secretion from pancreatic β -cells under basal glucose conditions in a dose-dependent manner. This stimulatory activity was comparable to that of known insulin secretagogues which indicates a potent ability of EEAH to modulate β -cell function. Importantly, EEAH maintained its insulin-releasing capacity in the presence of tolbutamide and high extracellular potassium, suggesting that its mechanism of action may involve both K_{ATP} channel-dependent and -independent pathways. The suppression of EEAH-induced insulin secretion by diazoxide supports the involvement of K_{ATP} channel closure in its effect [42]. Furthermore, the reduction of insulin release by verapamil implicates voltage-dependent Ca^{2+} influx as a crucial mediator of EEAH action. The synergistic enhancement of insulin secretion with IBMX co-treatment implies the potential involvement of cAMP-mediated pathways [43]. Collectively, these findings demonstrate that EEAH stimulates insulin secretion through a multimodal mechanism, including closure of K_{ATP} channels, activation of voltage-dependent Ca^{2+} channels, and modulation of the cAMP signaling pathway.

The significant proliferative effect of EEAH on BRIN-BD11 β -cells at both lower and higher concentrations compared to control also represents a potentially therapeutically relevant finding for diabetes management. This proliferative response mirrored the effect of GLP-1, positive control which exhibits its action through established cAMP/PKA pathway activation [44]. This differential response indicates that bioactive compounds in EEAH, likely including polyphenols and flavonoids, may stimulate this action via this pathway [45]. EEAH may stimulate the proliferation of insulin-producing β -cells by activating key pathways such as PI3K/Akt, Wnt/ β -catenin, or JAK-STAT, which are known to be involved in this regenerative process [46,47].

The pathogenesis of diabetes mellitus is generally multifactorial, key contributions often coming from enzymatic starch hydrolysis mediated by α -amylase and α -glucosidase as well as subsequent glucose absorption and diffusion across the intestinal epithelium. In this context, the ethanol extract of unripe *Artocarpus heterophyllus* (EEAH) exhibited a significant concentration-dependent inhibitory effect on enzymatic starch degradation, suggesting it may have the potential to attenuate postprandial hyperglycemia. This inhibitory activity may be attributed to the presence of bioactive phytoconstituents such as flavonoids and phenolic compounds known for their capacity to suppress α -amylase activity [48–50]. Additionally, EEAH significantly also impeded glucose diffusion and intestinal absorption in a concentration-dependent manner, indicating its possibility in limiting glucose bioavailability in the gastrointestinal tract. These findings align with previous literature documenting the antidiabetic potential of plant-based extracts through enzymatic and absorptive interference mechanisms [51].

Obesity which is known as a critical predisposing factor in case of T2DM, is often linked to elevated levels of non-esterified fatty acids (NEFAs) being released from adipose tissue, notorious in impairing insulin sensitivity and disrupting pancreatic β -cell function [52]. In the present investigation, EEAH resulted in a significant improvement in oral glucose tolerance of high-fat-fed (HFF) obese mice. These findings are in concordance with earlier plant based anti-diabetic studies, demonstrating that *A. heterophyllus* and other phytochemical-rich plant extracts can enhance glycemic control and preserve β -cell functionality in HFF-induced rodent models [53].

In the present study, chronic oral administration of the EEAH also demonstrated a significant antihyperglycemic effect in high-fat-fed (HFF) diet-induced obese mice during the 60-day treatment period. Twice-daily administration of EEAH has effectively led to a time-dependent reduction in blood glucose levels, with the higher dose exerting an earlier and more sustained glycemic control. These findings indicate a dose-responsive antihyperglycemic potential of EEAH, comparable to the standard antidiabetic agent, glibenclamide, which consistently reduced blood glucose throughout the study duration. The glycemic-lowering effect of EEAH may be attributed to its capacity to enhance pancreatic β -cell responsiveness, potentiate insulin secretion, and possibly modulate insulin signaling pathways [54]. These observations align with a previous study of *A. heterophyllus*, where similar blood glucose lowering trend was observed [54]. In both studies glibenclamide was used as a positive control that lowers blood glucose by binding to SUR1 on pancreatic β -cells, closing K_{ATP}

channels, leading to calcium influx and stimulating insulin secretion [56]. Therefore, the bioactive compounds present in *A. heterophyllum* might be modulating through this pathway as it showed a comparable glucose lowering trend compared to control.

EEAH administration was also associated with a gradual and sustained reduction in body weight in obese mice, indicating its potential role in ameliorating obesity-linked metabolic disturbances. Excessive caloric intake combined with inadequate energy expenditure disrupts lipid metabolism, leading to obesity and associated metabolic disorders like diabetes and non-alcoholic fatty liver disease (NAFLD). The effect on body weight by EEAH may reflect improved energy homeostasis and reduced adiposity, potentially through modulation of lipid metabolism and appetite regulation [57,58]. The presence of polyphenol also contributes to this appetite suppression and weight loss [59]. In line with this, EEAH also significantly improved both food and fluid intake patterns, which are typically disrupted in diabetic and obese states. These observations are consistent with earlier reports on *A. heterophyllum* [60].

Both tested doses of EEAH showed a marked increase in HDL-cholesterol levels while displayed a concurrent decrease in total cholesterol, LDL-cholesterol, and triglyceride levels. This lipid-modulating potential may be attributed to the extract's ability to enhance lipoprotein catabolism and promote reverse cholesterol transport, aligning with previous findings on plant-derived compounds with antihyperlipidemic activity [61]. The observed increase in HDL may further be supported by improved hormonal regulation, potentially involving thyroid-related pathways that facilitate the hepatic clearance of LDL [62].

EEAH treatment over 60 days resulted a significant improvement in gastrointestinal motility at higher dose. This suggests that EEAH may be helpful in reducing glucose absorption by accelerating transit time through the gastrointestinal tract. The increased motility could limit the duration available for nutrient absorption, contributing to the extract's antihyperglycemic activity [63]. Disaccharides like sucrose require breakdown into monosaccharides for absorption due to the absence of specific carriers in the GI tract; thus, the increased sucrose content following EEAH treatment likely reflects reduced sucrose digestion [64]. Such effects are likely linked to the presence of dietary fibers or bioactive compounds within EEAH that modulate gut function, similar to other plant-based treatments known to influence glucose regulation via digestive mechanisms [63,64].

Phytochemical analysis of the ethanol extract of unripe *A. heterophyllum* (EEAH) revealed the presence of several bioactive constituents, including alkaloids, tannins, flavonoids, saponins, steroids and terpenoids. Tannins are known to improve glucose uptake, block adipocyte differentiation, inhibit inflammation, oxidative stress and improve insulin sensitivity [65,66]. Saponins, particularly steroidal types, have been reported to mimic insulin action and improve glucose metabolism by modulating activity of adipokines [67]. Flavonoids, including well-studied compounds such as catechin and epicatechin, exert antioxidant effects and improve glycemic control by targeting pathways involved in insulin resistance and glucose homeostasis [68]. Alkaloids and terpenoids also play significant roles in modulating carbohydrate metabolism, enhancing GLUT-4 translocation and increasing pancreatic β -cell function [69]. The presence of these diverse phytochemicals in EEAH suggests a multifaceted mechanism underlying its antidiabetic potential, warranting further investigation into their individual and combined therapeutic contributions.

5. Conclusions

This study highlights the therapeutic potential of the ethanol extract of unripe *A. heterophyllum* (EEAH) as a promising candidate for the management of type 2 diabetes mellitus. EEAH demonstrated significant insulinotropic, antihyperglycemic and hypolipidemic effects, improved gastrointestinal motility, and contained a diverse array of phytochemicals known to modulate glucose metabolism, insulin sensitivity, and lipid profiles. These findings suggest a multimodal mechanism of action including enhancement of insulin secretion, modulation of nutrient absorption, and possible interaction with signaling pathways involved in metabolic regulation. Given the complex interplay of phytochemicals within EEAH and their broad biological targets, future research

should prioritize the isolation and characterization of bioactive phytomolecules responsible for the observed antidiabetic activity and the elucidation of their detailed mechanisms of action, ultimately paving the way for the development of targeted and effective therapeutic strategies. To further unravel these complex interactions, subsequent investigations using multi-omics approaches are essential. These integrative studies will provide a comprehensive understanding of the molecular mechanisms underpinning EEAH's antidiabetic action and aid in identifying specific biomarkers of efficacy and safety, ultimately supporting the development of EEAH as a standardized, evidence-based nutraceutical or adjunct therapy for diabetes management in humans.

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Institutional Review Board Statement: Animal experiments for this study were ethically approved by the Institutional Review Board (IRB) at Independent University, Bangladesh (IUB) on September 1, 2022. All procedures adhered strictly to national and international animal welfare guidelines, including the Animal Welfare Act 2019 of Bangladesh, the UK Animals (Scientific Procedures) Act 1986, and EU Directive 2010/63/EU. Experiments were conducted under UK Home Office Animal license PIL1822, ensuring high standards of animal care and minimizing distress.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data generated and analyzed during this study are not publicly accessible due to ethical or legal constraints. Requests for access to the data can be directed to the corresponding author and will be considered on a reasonable basis.

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