



Insulin secretory actions of ethanolic extract of *Acacia arabica* bark in high fat-fed-diet induced obese type 2 diabetic rats

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1 **Insulin secretory actions of ethanolic extract of *Acacia arabica* bark in high fat fed diet**
2 **induced obese type 2 diabetic rats**

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30

31 **Abstract**

32 *Acacia arabica* commonly known as “babul” has been widely used for the treatment of
33 numerous diseases, including diabetes due to their potential pharmacological actions. The
34 aim of the present study was to investigate the insulinotropic and anti-diabetic properties of
35 ethanol extract of *Acacia arabica* (EEAA) bark through *in vitro* and *in vivo* studies in high
36 fat-fed (HFF) rats. EEAA at 1.6-5000 µg/mL significantly increased ($p < 0.05-0.001$) insulin
37 secretion with 5.6 mM and 16.7 mM glucose, respectively from clonal pancreatic BRIN
38 BD11 β-cells. Similarly, EEAA at 10-40 µg/mL demonstrated a substantial ($p < 0.05-0.001$)
39 insulin secretory effect with 16.7 mM glucose from isolated mouse islets, with a magnitude
40 comparable to 1 µM glucagon-like peptide-1 (GLP-1). Diazoxide, verapamil, and calcium-
41 free conditions decreased insulin secretion by 25-26%. The insulin secretory effect was
42 further potentiated ($p < 0.05-0.01$) with 200 µM isobutylmethylxanthine (IBMX; 1.5-fold),
43 200 µM tolbutamide (1.4-fold), and 30 mM KCl (1.4-fold). EEAA at 40 µg/ml, induced
44 membrane depolarization and elevated intracellular Ca^{2+} as well as increased ($p < 0.05-0.001$)
45 glucose uptake in 3T3L1 cells and inhibited starch digestion, glucose diffusion, dipeptidyl
46 peptidase-IV (DPP-IV) enzyme activity, and protein glycation by 15-38%, 11-29%, 15-64%
47 and 21-38% ($p < 0.05-0.001$) respectively. In HFF rats, EEAA (250 mg/5 ml/kg) improved
48 glucose tolerance, plasma insulin, and GLP-1 levels, and lowered DPP-IV enzyme activity.
49 Phytochemical screening of EEAA revealed the presence of flavonoids, tannins and
50 anthraquinone. These naturally occurring phytoconstituents may contribute to the potential
51 anti-diabetic actions of EEAA. Thus, our finding suggests that EEAA, as a good source of
52 anti-diabetic constituents, would be beneficial for type 2 diabetes patients.

53

54 **Keywords:** Type 2 diabetes, obesity, glucose, Insulin, phytoconstituents, GLP-1

55 **1. Introduction**

56 Diabetes-related mortality has alarmingly escalated in response to the rising prevalence of the
57 disease globally [1]. The initial manifestation of diabetes is the impediment to glucose uptake
58 in muscles due to insulin resistance, which causes excessive blood glucose and abnormal
59 accumulation at various cellular locations leading to hyperglycaemia [2]. Inadequate insulin
60 production and impaired muscle glucose uptake result in significantly critical complications
61 such as nephropathy, retinopathy, and neuropathy, as well as the production of superoxide
62 free radicals due to hyperglycaemia-induced protein glycation [3, 4]. Additionally, the
63 correlation between obesity and diabetes has an effect on several organ systems and is linked
64 to various cardiovascular diseases [5]. The development of atherosclerosis and a high
65 mortality rate, as found in type 2 diabetic patients, are particularly linked with an altered lipid
66 profile or dyslipidemia [6]. It has been found that 90% of individuals with type 2 diabetes are
67 considered to be overweight or obese, making obesity a major variable risk factor for the
68 development of type 2 diabetes [5].

69 Proper nutrition, weight maintenance, and regular physical activity are necessary to keep
70 glycaemic levels under control [7]. In addition, single or combined synthetic oral antidiabetic
71 medicines such as biguanides, sulfonylureas, DPP-IV inhibitors, thiazolidinediones,
72 disaccharidase inhibitors, GLP-1 and GIP analogs, or/and insulin can be employed as insulin
73 secretagogues/insulinotropic agents for the management of diabetes mellitus [8]. These
74 synthetic drugs, however, present various adverse effects including hypoglycemia, weight
75 gain, GIT disorders, hypersensitivity reactions, liver, and kidney diseases, and are often
76 unavailable and inaccessible to people residing in rural areas [9-11]. Therefore, to overcome
77 the drawbacks of synthetic medicines, it is crucial to search for alternative medications that
78 are mostly derived from natural sources such as medicinal plants and animal derived peptides
79 [9].

80 From the beginning of civilization, medicinal plants have been considered an excellent source
81 of therapeutics owing to their plethora of health benefits. Herbal medicines have long been
82 used to cure a wide range of ailments due to the presence of numerous bioactive
83 phytoconstituents that exhibit various pharmacological actions, and their proven efficacy,
84 lower incidence of adverse effects in clinical studies, and affordability have encouraged many
85 medical professionals to practice them in practical life [9, 12]. Over 12,000 species of
86 medicinal plants have been identified to exhibit insulin-releasing and glucose-lowering action

87 [12]. The majority of these plants contain several classes of phytoconstituents such as
88 flavonoids, alkaloids, carotenoids, terpenoids, steroids, tannins, saponins, phenolic acids, and
89 glycosides [13, 14]. The antidiabetic activity of these compounds is generally attributed to
90 improvement in pancreatic β -cell function by increasing insulin secretion, decreasing
91 intestinal glucose absorption, or facilitating metabolism [15]. Thus, pure compounds of
92 medicinal plants or their crude extracts can be formulated as dietary supplements or
93 antidiabetic therapy to aid in the treatment of diabetes mellitus.

94 *Acacia arabica*, popularly known as Babul, is a tree belonging to the family of *Leguminosae*
95 and has been used in traditional medical practice for centuries [14]. Nearly all of its parts
96 including the bark, gum, leaves, roots, flowers, and pods are used as medicines. *Acacia*
97 *arabica* is well known around the world as a multipurpose tree and is used to treat bleeding
98 disorders, prolapse, leucorrhea, gastrointestinal disorder, diarrhea, constipation, and diabetes
99 in traditional medical practice [16]. In *Ayurvedic* medicine, the gum of *Acacia arabica* is
100 extensively utilized as a dietary supplement to manage diabetes [13]. Pharmacological
101 studies have shown that *Acacia arabica* has antioxidant, antidiabetic, antihypertensive,
102 antispasmodic, antibacterial, and antifungal properties [16]. A recent study conducted on
103 obese high-fat fed rats indicated that the hot water extract of *Acacia arabica* inhibits glucose
104 absorption, DPP-IV enzyme activity and improves β -cell function [13]. However, although
105 *Acacia arabica* is considered to exert glucose-lowering effects, only a few studies have been
106 conducted to assess its impact on insulin secretion and action [17]. Thus, the current
107 experiment was carried out to investigate the *in vitro* and *in vivo* antidiabetic effects of
108 ethanol extract of *Acacia arabica* (EEAA) bark to elucidate its mode of action in the
109 management of type 2 diabetes.

110 **2. Materials and methods**

111 **2.1. Collection and preparation of plant extracts**

112 *Acacia arabica* bark was collected and identified by a taxonomist at the Bangladesh National
113 Herbarium and assigned the accession number 43756 [13]. The obtained barks were rinsed,
114 air-dried, and then ground to a fine powder. The dry powdered (200g) bark was macerated in
115 1 L of 80% ethanol and agitated in an orbital shaker at a speed of 550 rpm for 48–72 h. The
116 filtrate was separated using Whatman no.1 filter paper and then dried off using a rotary
117 evaporator (BibbyRE-200, Sterilin Ltd., Newport, UK). The gummy residue was freeze-dried

118 in a freeze-dryer (Varian 801 LY-3-TT, Lexington, MA, USA) and preserved at 4°C until
119 further assayed [8].

120 **2.2. *In vitro* insulin-release studies using BRIN-BD11 cells**

121 Clonal pancreatic BRIN-BD11 β -cells were used for examining the insulin-releasing effects
122 of EEAA *in vitro*. EEAA or insulin modulators in the presence or absence of glucose (1.1,
123 5.6, or 16.7 mM) were incubated with BRIN-BD11 cells in a CO₂ incubator at 37°C for 20
124 min. The effects of EEAA in the presence of insulin secretagogues or inhibitors, such as
125 tolbutamide (a sulphonylurea and K_{ATP} channel blocker), diazoxide (a K_{ATP} channel opener),
126 verapamil (a voltage-dependent Ca²⁺ channel blocker), IBMX (a phosphodiesterase
127 inhibitor), 30 mM KCl, and 10 mM alanine, were studied in order to determine the insulin-
128 releasing pathways activated by EEAA. Membrane depolarization and Ca²⁺ influx is induced
129 by KCl and alanine. Alanine mostly accomplishes this via co-transport with Na⁺ and
130 metabolism with the generation of ATP [18, 19].

131 **2.3. Insulin-release studies using isolated mouse islets**

132 The impact of EEAA on insulin release was also investigated by using isolated mouse islets.
133 Pancreatic tissue of mice (40–50 gm, b.w.) was digested with collagenase P obtained from
134 *Clostridium histolyticum* (Sigma-Aldrich, Dorset, UK) to extract the islets. Islets were
135 cultured for 48-72 h in a CO₂ incubator at 37°C. Further islets were incubated with 1.4 and
136 16.7 mM glucose for 1 h, with or without EEAA, alanine, and GLP-1 respectively.
137 Centrifugation was used to separate the supernatants, which were stored at -20°C for
138 radioimmunoassay to measure the insulin concentration [20]. An acid-ethanol extraction
139 method was employed to measure the insulin content of islet cells [21].

140 **2.4. Membrane potential and intracellular calcium ([Ca²⁺]_i) concentration**

141 We used a Fluorometric Imaging Plate Reader (FLIPR) Membrane Potential and [Ca²⁺]_i
142 assay kit (Molecular Devices, Sunnyvale, CA, USA) to determine the intensity of membrane
143 depolarization and [Ca²⁺]_i in BRIN-BD11 cells treated with the EEAA. BRIN-BD11 cells
144 were seeded in 96-well plates and kept overnight in a CO₂ incubator at 37°C for adherence.
145 After the medium was withdrawn, the cells were pre-incubated with 100 μ L of 5.6 mM
146 glucose KRB buffer at 37°C for 10 min. Following the addition of 100 μ L of FLIPR
147 membrane potential or calcium dye, the cells were incubated at 37°C for 60 min. FlexStation
148 3 (Molecular Devices, Sunnyvale, CA, USA) was used to measure the fluctuations in signal

149 intensity. Depolarising concentrations of KCl (30 mM) and alanine (10 mM) were employed
150 as positive controls [8, 13].

151 **2.5. Glucose uptake**

152 The effect of EEAA on cellular glucose uptake was assessed using adipocytes produced from
153 3T3L1 fibroblast cells. The cells were treated with the EEAA and kept in a CO₂ incubator at
154 37°C for half an hour in the presence or absence of 100 nM insulin. The incubation was
155 continued with 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose, 2-NBDG
156 (50 nM) for an additional 5 min. Coverslips were fixed to the slides after the cells were rinsed
157 with ice-cold PBS. Using a fluorescent microscope (10× magnification), magnified images of
158 the fluorescence intensity were taken to evaluate the glucose uptake [13, 19].

159 **2.6. Glycation of insulin**

160 The impact of EEAA on insulin glycation was examined as previously described [22]. To
161 conduct the experiment, D-glucose (246.5 mM) was incubated with human insulin (1 mg/mL)
162 and NaBH₃CN (0.0853 gm/mL) with or without the EEAA treatment. After 24 h of
163 incubation, the reaction was stopped by the addition of 0.5 M acetic acid. Measurements of
164 glycated and non-glycated insulin were completed using reverse-phase HPLC [8].

165 **2.7. DPP-IV enzyme activity *in vitro***

166 Using a fluorometric technique, the effects of EEAA on the DPP-IV enzyme activity were
167 studied *in vitro*. The 96-well black-walled, clear-bottomed Greiner microplates containing 8
168 mU/ml of DPP-IV enzyme and 200 μM of Gly-Pro-AMC substrate were used to measure the
169 enzyme activity as previously described [23]. Variations in fluorescence were monitored
170 using the Flex Station 3 (Molecular Devices) with excitation and emission wavelengths at
171 370 and 440 nm with a 2.5 nm slit width, respectively [24].

172 **2.8. Starch digestion**

173 To investigate the impact on starch digestion, EEAA or acarbose was incubated with 100 mg
174 of starch solution (Sigma-Aldrich, St. Louis, MO, USA). After dilution, the mixture was
175 treated with thermostable α-amylase (0.01%) from *Bacillus leicheniformis* and
176 amyloglucosidase (0.1%) from *Rhizopus* mold (Sigma-Aldrich, St. Louis, USA). The GOD-
177 PAP method (Randox GL 2623) was implemented to further analyze the final samples
178 aliquoted for the measurement of glucose concentration [8].

179 **2.9. Glucose diffusion *in vitro***

180 A cellulose ester dialysis tube (CEDT) (20 cm 7.5 mm, Spectra/Por®CE layer, MWCO:
181 2000, Spectrum, The Netherlands) was used to test the effects of EEAA on glucose diffusion
182 *in vitro*. To perform the experiment, the tubes were filled with 2 mL of 0.9% NaCl and 220
183 mM glucose in the presence or absence of the EEAA (0.2-25 mg/mL), and the ends were
184 tightly sealed. Afterward, the CEDT was put into 50 mL Falcon conical tubes (Orange
185 Scientific, Orange, CA, USA) containing 0.9% NaCl (45 mL) solution and agitated in an
186 orbital shaker at $20 \pm 2^\circ\text{C}$. The samples were collected 24 h later, and the GOD-PAP method
187 (Randox GL 2623), as previously reported [18, 25], was used to analyze the aliquoted
188 samples for the detection of glucose diffusion and absorption.

189 **2.10. Animals**

190 The experiments were carried out on 6-8 weeks aged male Sprague Dawley rats (Envigo UK)
191 weighing between 200–250 g. Prior to the experiments, the animals were given access to a
192 high-fat diet for 6-8 weeks consisting of 20% protein, 45% fat, and 35% carbohydrates with a
193 total energy content of 26.15 KJ/g. A standard diet of 10% fat, 30% protein, and 60%
194 carbohydrates with a metabolizable energy content of 12.99 KJ/g was fed to normal rats
195 (Trouw Nutrition, Cheshire, UK). The animals were accommodated in regulated conditions
196 of $25 \pm 0.5^\circ\text{C}$ temperature and 65–70% humidity and an automated 12 h dark-light cycle
197 system was installed in the animal house to maintain a day-night circadian rhythm. Before
198 performing the experiments, the fasting blood glucose was determined in HFF diet rats in
199 order to distinguish each group. HFF diet-induced obese type 2 diabetic rats were defined as
200 those with fasting blood glucose levels that were higher than normal (>6.0 mmol/L). The
201 groups were divided in the following manner:

202 Group 1: Lean control (Saline)

203 Group 2: High fat-fed diet control (Saline)

204 Group 3: High fat fed diet + EEAA (250 mg/5 ml/kg)

205 Group 4: High fat fed diet + sitagliptin (10 $\mu\text{mol}/5$ ml/kg)

206 Group 5: High fat fed diet + vildagliptin (10 $\mu\text{mol}/5$ ml/kg)

207 **2.11. Oral glucose tolerance**

208 To assess the effects of EEAA on oral glucose tolerance, the high-fat-fed rats were starved
209 overnight and oral gavage of glucose (18 mmol/kg, body weight (b.w.)) with or without the
210 treatment (250 mg/5 mL/kg, b.w.) were given to both normal and HFF rats. Samples of blood
211 were obtained using heparinized microvessel blood collection tubes (Sarstedt, Numbrecht,
212 Germany) from the tip of the tail at 0 min before and at 30, 60, 120, and 180 min after the
213 glucose/drug administration. Followed by centrifugation at 12,000 rpm at 4°C for 5 min, the
214 plasma was separated and stored at -20°C until further insulin assay. Blood glucose levels
215 were measured using Ascencia Contour glucose meters (Bayer, Newbury, UK) and insulin
216 levels were measured by a dextran-charcoal radioimmunoassay [8, 13].

217 **2.12. DPP-IV enzyme activity *in vivo***

218 A fluorometric assay was employed to study the impact of EEAA on plasma DPP-IV enzyme
219 activity in high-fat-fed rats. Blood samples were taken from overnight fasted HFF rats before
220 (at 0 min) and after (30, 60, 120, and 180 min) oral administration of EEAA (250 mg/5
221 mL/kg), the DPP-IV inhibitors, vildagliptin (10 µmol/5 mL/kg), and sitagliptin (10 µmol/5
222 mL/kg) or saline (5 mL/kg). Plasma serum was separated by centrifugation and the samples
223 (10 µL) were incubated in 96-well microplates with 40 µL of Tris-HCl (100 mM) buffer (pH
224 7.4) and 50 µL of Gly-Pro-AMC (200 µM) substrate for 30 min at 37°C. Hydrolysis of the
225 fluorogenic substrate bonds (H-Gly-Pro) conjugated to the AMC group (H-Gly-Pro-AMC) by
226 the DPP-IV enzyme in the blood serum caused the formation of the fluorescent 7-Amino-4-
227 Methyl Coumarin (AMC). As mentioned above in the section on *in vitro* DPP-IV enzyme
228 activity, the fluorescence changes were monitored using FlexStation 3. Plasma samples
229 collected at 60 min were used to determine levels of active GLP-1 (7-36) using a GLP-1
230 (Active) ELISA Kit (EGLP-35K, Merck Millipore, Dorset, UK) [21].

231 **2.13. Phytochemical screening**

232 The EEAA was subjected to phytochemical screening to determine the presence or absence
233 of phytochemicals including glycosides, reducing sugars, flavonoids, alkaloids, terpenoids,
234 tannins, and anthraquinones as per previous methods [21, 26-28].

235 **Alkaloids:** Alkaloid testing was done by acidifying 2 mL of the EEAA in dilute hydrochloric
236 acid to which 1 mL of Dragendroff's reagent was added. The precipitate's color change from
237 orange to crimson red confirmed the presence of alkaloids [26].

238 **Flavonoids:** The presence of flavonoids was tested by mixing 4 mL of the EEAA with 1.5
239 mL methanol, which was then heated. Upon the addition of magnesium metal together with
240 2-3 drops of hydrochloric acid, the solution's color changed to pink indicating a positive
241 result [26].

242 **Tannins:** To test for tannins, a few drops of 10% lead acetate were added to 2 mL of the
243 EEAA. The formation of white sediment suggested the presence of tannins [21].

244 **Terpenoids:** Terpenoids were tested by dissolving 1 g of the EEAA in 2mL of chloroform to
245 which 3mL of strong sulphuric acid was carefully added to form a layer; the presence of
246 terpenoids was indicated by a reddish-brown coloration on the interface [27].

247 **Glycosides:** To test for glycosides, 1 mL of the EEAA was combined with a few drops of
248 glacial acetic acid, and ferric chloride to form a mixture, to which concentrated sulfuric acid
249 was added afterward. The presence of glycoside was evidenced by the visualization of a blue-
250 green color [21].

251 **Anthraquinone:** To test for anthraquinones, a dry test tube was filled with about 0.5 g of the
252 EEAA, 5mL of chloroform, and was shaken vigorously for 5 min. After filtering the mixture,
253 an equal amount of 10% ammonia solution was mixed into the filtrate and the presence of
254 anthraquinone was confirmed upon the formation of pink-violet or red color in the lower
255 layer [28].

256 **Reducing sugars:** Reducing sugars were detected by mixing 1 mL of the EEAA, 1mL of
257 distilled water, and a few (4-6) drops of Fehling's reagent, and the mixture was heated. The
258 formation of a reddish-brown color confirmed the presence of reducing sugars [21].

259 **2.14. Statistical analysis**

260 All statistical analysis and data interpretation were conducted using Graph Pad prism 5. The
261 unpaired Student's t-test (nonparametric, with two-tailed p-values) and one-way or two-way
262 ANOVA with Sidek post hoc tests were used to analyze the data. The significance threshold
263 was set at $p < 0.05$, and values were presented as the mean \pm SEM.

264 **3. Results**

265 **3.1. EEAA and insulin release from BRIN-BD11 cells**

266 Concentration-dependent (1.6-5000 $\mu\text{g/mL}$) insulin-releasing effects of EEAA are presented
267 in Figure 1 (A, B). The basal rate of insulin release at 5.6 mM glucose (Figure 1A) from

268 BRIN-BD11 cells was 0.89 ± 0.02 ng/ 10^6 cells/20 min. The positive control, alanine (10mM)
269 increased the insulin-releasing rate to 4.45 ± 0.54 ng/ 10^6 cells/20 min (Figure 1A; $P < 0.001$; n
270 = 8). EEAA (40-5000 μ g/mL) increased insulin release from 2.05 ± 0.21 to 7.1 ± 1.1 ng/ 10^6
271 cells/20 min (Figure 1A; $p < 0.05-0.001$) with 5.6 mM glucose. In the presence of 16.7 mM
272 glucose (Figure 1B), the basal insulin rate was 1.53 ± 0.12 ng/ 10^6 cells/20 min and with the
273 depolarising concentration of KCl (30 mM), it was increased to 8.78 ± 0.69 ng/ 10^6 cells/20
274 min ($p < 0.001$). Additionally, EEAA with 16.7 mM glucose enhanced the release of insulin
275 from 3.05 ± 0.31 to 8.86 ± 1.58 ng/ 10^6 cells/20 min (Figure 1B; $p < 0.05-0.001$) in a dose-
276 dependent manner (40-5000 μ g/mL). An increase in LDH release was observed with
277 increasing extract concentrations, however, there was no effect on cellular viability at lower
278 doses (Data not shown).

279 **3.2. EEAA and insulin release from isolated mouse islets**

280 The insulin-releasing effects of EEAA from isolated mouse islets are illustrated in figure 1C.
281 At 16.7 mM, the basal rate of insulin secretion from isolated mouse islets was 7.15 ± 0.78
282 ng/ 10^6 cells/20 min. EEAA showed a significant increase (Figure 1C; $p < 0.05-0.001$) in
283 insulin secretion from 11.29 ± 1.02 to 16.71 ± 1.24 with 16.7 mM glucose in a concentration-
284 dependent manner (10-40 μ g/mL). As a positive control alanine (10 mM) and GLP-1 (10^{-6} &
285 10^{-8} M) significantly stimulated (Figure 1C; $p < 0.001$) the release of insulin from $12.27 \pm$
286 0.94 to 27.53 ± 1.42 at 16.7 mM glucose. However, the increase in insulin secretion by
287 EEAA was lower than the GLP-1 (10^{-6} & 10^{-8} M) in presence of 16.7mM glucose.

288 **3.3. EEAA and known modulators of insulin release and, inhibitors or absence of** 289 **extracellular calcium**

290 EEAA (40 g/mL) bark was treated with established insulin releasing modulators to assess
291 their insulin secretory actions (Figure 1E). The release of insulin was significantly augmented
292 (Figure 1E) with modulators such as 16.7 mM glucose ($p < 0.05$), IBMX ($p < 0.001$), and
293 tolbutamide ($p < 0.001$). EEAA resulted in a significant rise in insulin secretion by 1.4-fold
294 when combined with a depolarizing concentration of 30 mM KCl ($p < 0.01$; Figure 1E).
295 Insulin release activity was further increased following co-treatment of EEAA with IBMX
296 (by 1.5-fold; $p < 0.05$) and tolbutamide (by 1.3-fold; $p < 0.01$). In the presence of K^+ channel
297 activator diazoxide (300 μ M), L-type voltage-dependent Ca^{2+} channels blocker verapamil (50
298 μ M), and free extracellular Ca^{2+} , the insulin-releasing rate was attenuated by 25-26%
299 respectively (Figure 1; E, F).

300 **3.4. EEAA and membrane depolarization and, $[Ca^{2+}]_i$ in BRIN-BD11 cells**

301 Depolarization of membrane potential and intracellular calcium ($[Ca^{2+}]_i$) concentration in
302 clonal BRIN-BD11 cells were assessed (Figure 2A & B). A significant induce in membrane
303 depolarization (94%; Figure 2A) and an increase in intracellular calcium concentration
304 ($[Ca^{2+}]_i$) (80%; Figure 2B) were observed in incubation with KCl (30 mM) and alanine (10
305 mM). EEAA at a concentration of 40 μ g/mL induced ($p < 0.001$) depolarization of membrane
306 potential by 87% (Figure 2A) followed by an increase in $[Ca^{2+}]_i$ concentration by 69%
307 (Figure 2B).

308 **3.5. EEAA and glucose uptake and insulin action**

309 The glucose analogue 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose (2-
310 NBDG) fluorescent hexose was used to assess the effect of EEAA on glucose uptake and
311 insulin action using 3T3L1 differentiated adipocyte cells (Figure 2C-G). In the microscopic
312 fluorescence analysis, EEAA enhanced glucose uptake significantly with ($p < 0.05$; Figure
313 2G) or without ($p < 0.001$; Figure 2G) insulin (100 nM) when compared to the control.
314 Insulin alone stimulated glucose uptake by 2.6-fold ($p < 0.01$; Figure 2G) compared to the
315 control.

316 **3.6. EEAA and glycation of insulin**

317 EEAA showed a significant inhibitory effect on insulin glycation (Figure 1D). EEAA caused
318 a 21.6% inhibition at the concentration of 40 μ g/mL ($p < 0.05$; Figure 1D), while the effect
319 increased to 38.4% at 200 μ g/mL ($p < 0.01$; Figure 1D). With 44 mM aminoguanidine, the
320 inhibition of insulin glycation increased to 80% ($p < 0.001$; Figure 1D).

321 **3.7. EEAA and starch digestion**

322 The effects of EEAA on starch digestion are shown in Figure 2H. Acarbose (1mg/mL),
323 employed as a positive control, decreased enzymatic glucose liberation from starch by 72%
324 (data not shown). EEAA at a dose of 40–1000 μ g/mL showed 14%-38% inhibitory activity (p
325 < 0.05 - 0.001) in glucose liberation from starch.

326 **3.8. EEAA and glucose diffusion *in vitro***

327 EEAA showed significant inhibitory effects on glucose diffusion and absorption (Figure 2I)
328 over a 24 h incubation period compared to the control group. Doses of 0.2-5 mg/mL EEAA
329 showed an 11% -29% inhibitory effect ($p < 0.05$ - 0.001 ; Figure 2I).

330 **3.9. EEAA and DPP-IV enzyme activity *in vitro***

331 Effects of EEAA on DPP-IV enzyme activity (Figure 3A) were evaluated by an *in vitro*
 332 fluorometric method. An established DPP-IV inhibitor, sitagliptin (10 μ M) reduced DPP-IV
 333 enzyme activity by 97.5% (Data not shown). At a dose of 40-5000 μ g/mL EEAA, the
 334 inhibition of the DPP-IV enzyme increased by 15%-64% ($p < 0.01-0.001$; Figure 3A).

335 **3.10. EEAA and oral glucose tolerance and plasma insulin**

336 Oral administration of EEAA (250 mg/5 mL/kg; body weight) in conjunction with glucose
 337 (18 mmol/5 mL/kg; body weight) showed a significant ($p < 0.05$; Figure 3B) reduction in
 338 blood glucose at 30 and 60 mins compared to the HFF diet control. EEAA also increased
 339 plasma insulin concentrations at 30 mins ($p < 0.05$; Figure 3C) as compared to HFF rats.

340 **3.11. EEAA and DPP-IV enzyme activity and active GLP-1 (7–36) Levels**

341 EEAA (250 mg/5 mL/kg; body weight) reduced DPP-IV enzyme activity ($p < 0.05-0.01$;
 342 Figure 3D) at 60 and 120 mins compared to the high-fat-fed diet control group. The standard
 343 drugs sitagliptin and vildagliptin (10 μ mol/5 mL /kg), showed consistent ($p < 0.001$; Figure
 344 3D) reduction in DPP-IV enzyme activity with respect to time (30 to 180 mins). Oral gavage
 345 of EEAA (250 mg/5 mL/kg; body weight) increased the level of plasma active GLP-1 (7-36)
 346 in the circulation by 28% ($p < 0.05$; Figure 3E) and this was increased to 83%-92% ($p <$
 347 0.001 ; Figure 3E) with sitagliptin (10 μ mol/5 mL/kg) and vildagliptin (10 μ mol/5 mL/kg),
 348 respectively.

349 **3.12. Phytochemical screening of EEAA**

350 To determine the presence of anticipated anti-diabetic phytochemicals, additional studies
 351 were done (Table 1). Flavonoids, tannins, terpenoids, glycosides, and anthraquinone were
 352 identified in EEAA (Table 1).

353 **Table 1: Preliminary phytochemical analysis of ethanol extract of *Acacia arabica* bark**

Group	Result
Alkaloids	–
Flavonoids	+
Tannins	+
Terpenoids	+
Glycosides	+
Anthraquinone	+

Reducing sugars —

354 The '+' sign denotes the presence of phytoconstituents whereas '-' sign denotes the absence
355 of phytoconstituents in EEAA. The tests were carried out three times (n=3).

356 4. Discussion

357 The gum of *Acacia arabica* is widely employed as an ethnomedicine due to its numerous
358 therapeutic benefits including glucose-lowering effects and has been scientifically proven to
359 possess anti-hyperglycaemic properties [29]. Previous studies reported that hot water extract
360 of *Acacia arabica* improves β -cell functions in HFF diabetic animal models [13]. However,
361 the molecular mechanism underlying EEAA's antidiabetic and insulinotropic activities has
362 yet to be detailed [30, 31]. The objective of the present study was to investigate insulin-
363 releasing and glucose-lowering actions of EEAA through *in vitro* and *in vivo* studies to
364 explore its underlying mechanism of action for the treatment of type 2 diabetes.

365 In this study, the insulinotropic effects of EEAA were explored using clonal pancreatic
366 BRIN-BD11 cells and isolated mouse islets where EEAA stimulated insulin release in a dose-
367 dependent manner. The mechanisms underlying the stimulation of insulin secretion were also
368 investigated using non-toxic concentrations of EEAA in the presence or absence of known
369 modulators of β -cell function. In response to 16.7mM glucose, EEAA stimulated basal
370 insulin secretion. The effects of tolbutamide and membrane-depolarizing concentrations of
371 KCl (30 mM) were examined to evaluate their effects in the absence and presence of EEAA.
372 It is known that the action of this sulphonylurea involves the closure of K_{ATP} channels,
373 depolarization of the plasma membrane, and stimulation of Ca^{2+} influx via the activation of
374 L-type voltage-dependent calcium channels [32]. In both conditions, EEAA increased insulin
375 release indicating the ability of EEAA to potentiate insulin secretion through various
376 mechanistic pathways including a direct effect on exocytosis or phosphatidylinositol (PI3) or
377 adenylate cyclase/cAMP pathway [33]. Additionally, the stimulatory activity of EEAA also
378 showed the involvement of ion channels in clonal pancreatic β -cells. The insulin-releasing
379 actions of EEAA were suppressed by the K_{ATP} -channel opener diazoxide, indicating that the
380 closure of K_{ATP} channels contributes to EEAA's insulinotropic action. These findings are
381 consistent with our observations using the L-type voltage-dependent Ca^{2+} channel blocker,
382 verapamil, which partially decreased EEAA-mediated insulin release, suggesting its
383 dependency on insulin release on the Ca^{2+} channel [34]. Examining the effects of Ca^{2+} free
384 buffer revealed a similar dependency on extracellular Ca^{2+} . The effects of the absence of Ca^{2+}
385 on insulin secretion were not fully abolished suggesting that EEAA is capable of both
386 inducing intracellular Ca^{2+} mobilization and Ca^{2+} entry. The direct observation of
387 intracellular Ca^{2+} in BRIN-BD11 cells also provided strong evidence for this finding.

388 Furthermore, the phosphodiesterase inhibitor, IBMX, also potentiated the insulin-releasing
389 effects of EEAA, indicating the involvement of the cAMP pathway [35].

390 Insulin plays a key role in the regulation of glucose disposal in peripheral tissues like skeletal
391 muscles, adipose tissues, and the liver [36]. Recent studies have shown that the stimulation of
392 glucose uptake by insulin via the insulin receptor substrate 1/phosphoinositol 3-kinase (IRS-
393 1/P3K) and GLUT4 translocation by muscular contraction or exercise by the activation of
394 AMPK is mediated by a distinctive intracellular signaling pathway [37]. Furthermore, as
395 skeletal muscle is the main location for using both glucose and fatty acids, insulin resistance
396 associated with type 2 diabetes is mostly found in this tissue [37]. In this present study, we
397 investigated EEAA's effects on glucose uptake in 3T3L1 adipocyte cells. It has been
398 observed that EEAA increases glucose uptake in 3T3L1 cells. Earlier investigations on
399 *Acacia arabica* showed the presence of kaempferol, quercetin, and gallic acids, which
400 stimulate AMP-activated protein kinase activity and increase GLUT4 translocation [38, 39].
401 A previous study has also demonstrated that quercetin promotes GLUT4 translocation by
402 concurrently increasing the phosphorylation of both AMPK and AKT which in turn resulted
403 in the stimulation of glucose uptake in skeletal muscle cells and adipose tissues [37, 40].
404 Therefore, the presence of flavonoids such as quercetin in EEAA may be responsible for
405 EEAA's potential to increase glucose transport in skeletal muscles and adipocyte cells via
406 activating signaling pathways [13].

407 Non-enzymatic glycosylation of structural proteins is speculated to be an important factor
408 contributing to the onset of diabetes associated complications [41]. It has been observed that
409 the glycation of insulin decreases its biological action. This reduced biological activity of
410 glycated insulin may be caused due to its decreased affinity for the insulin receptor, poor
411 insulin signaling, or it may function as a ligand for the receptor for advanced glycation end
412 products (RAGE), activating oxidative stress and pro-inflammatory pathways that result in
413 insulin resistance [42]. In our study, EEAA was found to decrease insulin glycation in a
414 concentration-dependent manner. In earlier investigations, *Acacia arabica* was reported to
415 contain well-known antioxidant constituents such as flavonoids, glycosides, quercetin, and
416 gallic acids [43]. Thus, the antiglycation effects demonstrated by the EEAA may be due to its
417 phytochemicals and antioxidant properties [44].

418 The effects of EEAA on α -amylase and α -glucosidase enzymes on glucose release from
419 starch following digestion were studied *in vitro*. Acarbose, an established α -glucosidase

420 inhibitor decreased glucose liberation significantly. The concentration-dependent inhibition
421 of glucose release from starch was observed with EEAA. Previous studies found that
422 flavonoids are very effective in reducing the α -amylase activity and slowing down starch
423 digestion [45]. It is also known that increased intake of dietary fiber helps to suppress
424 appetite. In addition, dietary fiber impedes stomach emptying and/or delays energy and
425 nutrient absorption which results in lower post-prandial glucose and lipid levels [46]. Results
426 from previous investigations have reported the high fibre content [47] in *Acacia arabica*
427 which may also be responsible for the postprandial glucose-lowering effects of EEAA due to
428 slower digestion and longer duration of nutritional absorption.

429 Several medicinal plants have been found to limit gastrointestinal glucose absorption, which
430 may be a factor in how effective they are at preventing hyperglycaemia [48]. There are
431 different mechanisms by which medicinal plants interfere with glucose absorption into cells
432 such as by decreasing the gastric emptying time and obstructing the absorption of glucose
433 from the intestine, inhibiting disaccharidase enzymes like α -amylase and α -glucosidase and
434 preventing the breakdown of carbohydrates, stimulating insulin release, inhibiting
435 gluconeogenesis or by enhancing the uptake of glucose into peripheral cells [49]. In this
436 current study, a simple *in vitro* dialysis-based model was utilized to examine the effects of
437 EEAA on glucose diffusion. Although this model used constant agitation to simulate
438 gastrointestinal movement, it has certain limitations because it does not directly compare the
439 timing of cellular mechanisms for glucose absorption in the gut with the time it takes for
440 glucose to completely diffuse from the dialysis tube (22–26 h). Our results have depicted that
441 EEAA demonstrates significant dose-dependent inhibition of the movement of glucose
442 through the dialysis membrane. These findings are in agreement with previous findings
443 conducted on alloxan-induced diabetic rats and rabbits which reported that a diet containing
444 *Acacia arabica* exhibits anti-hyperglycaemic activity [50, 51].

445 The progression of type 2 diabetes is linked to obesity. Obesity is characterized by the
446 presence of non-esterified fatty acids (NEFAs) released from adipose tissue, which
447 contributes to insulin resistance and β -cell dysfunction, resulting in type 2 diabetes. In our
448 present study, EEAA improved glucose tolerance and plasma insulin significantly in HFF
449 diet-induced obese rats. It was observed that the tannins present in *Acacia arabica* improves
450 the release of insulin from pancreatic β -cells and restored their functionality [17].
451 Furthermore, flavonoids such as quercetin, catechin, and kaempferol were also reported to

452 increase insulin secretion and improve glucose uptake, plasma insulin responses, and glucose
453 tolerance in mice [9, 13]. Therefore, it may be reasonable to assume that the anti-
454 hyperglycaemic effects of EEAA are attributable to these phytomolecules.

455 Several pharmaceutical methods have been developed to treat type 2 diabetes by focusing on
456 the development of oral DPP-IV inhibitors to block the degradation of the incretin hormones
457 GLP-1 and GIP [52]. In the current study, EEAA inhibited DPP-IV enzyme activity *in vitro*
458 in a concentration-dependent manner, which was consistent with our *in vivo* findings in HFF
459 rats. GLP-1 and GIP hormones play important roles in regulating insulin secretion and
460 management of type 2 diabetes by augmenting glucose-stimulated insulin secretion via the
461 cAMP signaling pathway [53, 54]. The combined effect of GLP-1 and GIP in stimulating
462 insulin secretion in a glucose-dependent manner, prolonging stomach emptying time, and
463 suppressing hunger, thus, significantly improving the management of postprandial
464 hyperglycaemia in particular [55]. EEAA also increased the levels of active GLP-1 (7–36) in
465 the bloodstream. Previous studies have shown that reduced levels of the antagonistic
466 metabolite and increased amounts of active GLP-1 can be achieved by inhibiting DPP-IV
467 enzyme activity, which could be beneficial in the treatment of impaired glucose tolerance and
468 type 2 diabetes. [56]. The flavonoids present in natural products and crude herbal extracts
469 have previously been found to exert promising DPP-IV enzyme inhibitory action which acts
470 via binding to the DPP-IV and causing a conformational shift that inhibits the active site of
471 the enzyme [57, 58]. Therefore, it may be reasonable to assume that the presence of
472 flavonoids in EEAA may be responsible for the DPP-IV enzyme inhibition and enhancement
473 of GLP-1 action to aid in the maintenance of glucose homeostasis.

474 Phytochemical screening of EEAA identified the presence of different classes of
475 phytochemicals including tannins, terpenoids, glycosides, anthraquinones, and flavonoids
476 such as kaempferol and quercetin [13] which are consistent with the results of earlier studies
477 [14]. Flavonoids have previously been observed to improve glucose homeostasis and β -cell
478 function in STZ-induced rats [59, 60]. It has also been documented that the antidiabetic effect
479 of flavonoids aid in the regulation of glucose absorption, insulin signaling, insulin secretion,
480 and adipose deposition [61]. Additionally, they target a number of molecules that are
481 involved in the regulation of various pathways such as stimulating the PLC/PKC and/or
482 cAMP/PKA signaling pathways in order to improve β -cell proliferation, and promote insulin
483 secretion [62], prevent cellular apoptosis via inhibition PI3K/Akt pathway [63], and lower

484 hyperglycaemia through regulating hepatic glucose metabolism [61]. Furthermore, flavonoids
485 have also been reported to prevent diabetes-associated microvascular complications such as
486 protecting from diabetic retinopathy by improving the retinal SIRT-1 pathway, alleviating
487 diabetic neuropathy via activation of Nrf-2/HO-1 and inhibition of nuclear factor K beta (NF-
488 κ B) as well as inhibition of advanced glycation end-products generation [64]. Findings from
489 previous studies have also shown that tannins promote the utilization of carbohydrates by
490 receptor cells to enhance glucose uptake via phosphorylation of the protein components
491 involved in the signaling cascade of insulin-mediated glucose transport, including the insulin
492 receptor (IR), Akt, and translocation of the glucose transporter 4 (GLUT 4) [65]. Previous *in*
493 *vitro* and *in vivo* studies reported that monoterpenes, known as terpenoid, exert the
494 antidiabetic effect by lowering the blood glucose levels, reducing TC, TG, and plasma
495 glucose, as well as improving impaired renal function [66]. Additionally, it has also been
496 reported that anthraquinones improve glucose tolerance, enhance glucose uptake in cells, and
497 improve glycaemic levels via several pathways including stimulation of PPAR- γ , inhibition
498 of α -glucosidase activity, and regulation of the AKT/GSK-3 β signaling pathway [67]. Hence,
499 it may be concluded that the presence of these phytochemicals in EEAA is responsible for its
500 insulinotropic and glucose-lowering effects. However, further research is certainly warranted
501 to corroborate this hypothesis.

502 **5. Conclusion**

503 To summarize, the current study has demonstrated that the anti-hyperglycaemic effects of
504 EEAA bark are linked to decreased intestinal glucose absorption and increased tissue glucose
505 utilization, which is facilitated by an increase in insulin release from clonal pancreatic β -cells
506 and isolated mouse islets. In addition to that, the decrease in DPP-IV enzyme activity
507 increased the amount of active GLP-1 (7-36) level in the systemic circulation. These effects
508 might be attributed to the presence of various bioactive constituents such as flavonoids,
509 tannins, terpenoids, and anthraquinones. As a result, we might speculate that *Acacia arabica*
510 could be used as a dietary supplement as well as a possible source of oral antidiabetic agents
511 to treat hyperglycaemia. However, further in-depth studies are needed to investigate the role
512 of *Acacia arabica* and its marker compounds in the prevention and management of type 2
513 diabetes in individuals.

514 **Data Availability**

515 All data are included in the manuscripts, and the identifiable participant information (PA) for
516 the data collections is included in the Author Contribution section.

517 **Competing Interests**

518 The authors declare that there are no competing interests linked with this manuscript.

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521 **Author Contribution**

522 P.A. and Y.H.A.A.-W. shared equal responsibility for the conception, design, and supervision
523 of the study; P.A., S.S.I., J.T.K., J.A.S. and S.A. carried out the experiments, analyzed the
524 data, assessed the results, and prepared the figures. The article was drafted by P.A., S.A.,
525 S.S.I. and J.T.K. and the revised manuscript was edited by P.A. and Y.H.A.A.-W. The
526 published version of the manuscript has been read and approved by all authors.

527 **Institutional Animal Care**

528 The Animal Welfare and Ethical Review Board (AWERB) at Ulster University approved the
529 use of animals for research in May 2018. The UK Home Office issued project/personal
530 license numbers PIL1822 and PPL2804 in May 2016 and February 2017, respectively, under
531 which the experiments were conducted. All experiments were performed in the Biomedical
532 and Behavioral Research Unit (BBRU) at Ulster University, Coleraine, U.K. in accordance
533 with the UK Act 1986 and EU Directive 2010/63EU, and necessary measures were taken to
534 make sure no animals were harmed throughout the course of the research. Samples of blood
535 were obtained from the cut tail tips of live animals; they were not executed.

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539 providing the laboratory facilities to conduct the studies.

540 **Abbreviations**

541	EEAA	Ethanol Extract of <i>Acacia arabica</i>
542	KCl	Potassium chloride
543	DPP-IV	Dipeptidyl peptidase-IV
544	HFF	High fat fed
545	GLP-1	Glucagon-like peptide-1
546	GIP	Glucose-dependent insulinotropic polypeptide
547	GIT	Gastrointestinal tract
548	K _{ATP}	Adenosine triphosphate-sensitive potassium channel
549	FLIPR	Fluorometric Imaging Plate Reader
550	KRB	Krebs-Ringer Bicarbonate
551	2 NBDG	2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose
552	PBS	Phosphate-buffered saline
553	HPLC	High performance liquid chromatography
554	Gly-Pro-AMC	Gly-Pro-7-Amino-4-Methyl-Coumarin
555	GOD/PAP	Glucose oxidase-phenol amino phenazone
556	CEDT	Cellulose ester dialysis tube
557	IRS	Insulin Receptor Substrate
558	PKB	Protein kinase B
559	GLUT-4	Glucose transporter type 4
560	AMPK	Adenosine monophosphate protein kinase
561	PLC/PKC	Phospholipase C (PLC)/protein kinase C
562	cAMP	cyclic Adenosine monophosphate
563	PKA	Protein kinase A

564	PI3K/Akt	Phosphoinositide 3-kinase/protein kinase B		
565	SIRT-1	Sirtuin 1		
566	NrF-2/HO-1	Nuclear factor erythroid-2 related factor 2/heme oxygenase		
567	NF-kB	Nuclear factor kappa B		
568	TC	Total cholesterol		
569	TG	Triglycerides		
570	PPAR- γ	Peroxisome proliferator-activated receptor- γ		
571	GSK-3 β	Glycogen	Synthase	Kinase-3 β

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777

778 **Figure title with Legends**

779 **Figure 1: Effects of ethanol extract of *Acacia arabica* (EEAA) bark on insulin secretion**
780 **from (A, B) clonal pancreatic BRIN BD11 β cells, as well as (C) pancreatic islets of**
781 **Langerhans, (D) protein glycation, (E) insulin secretion with known stimulators or**
782 **inhibitors and (F) with or without extracellular calcium.**

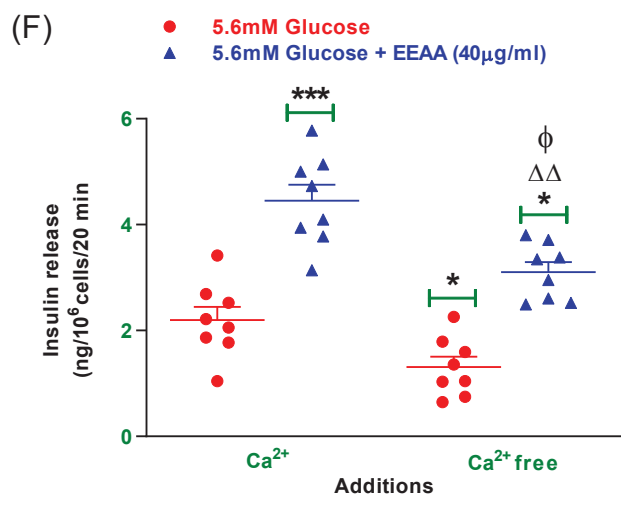
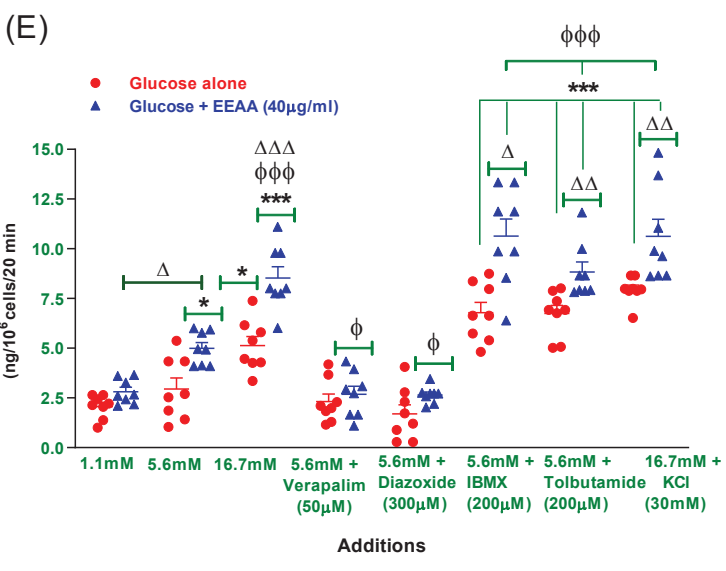
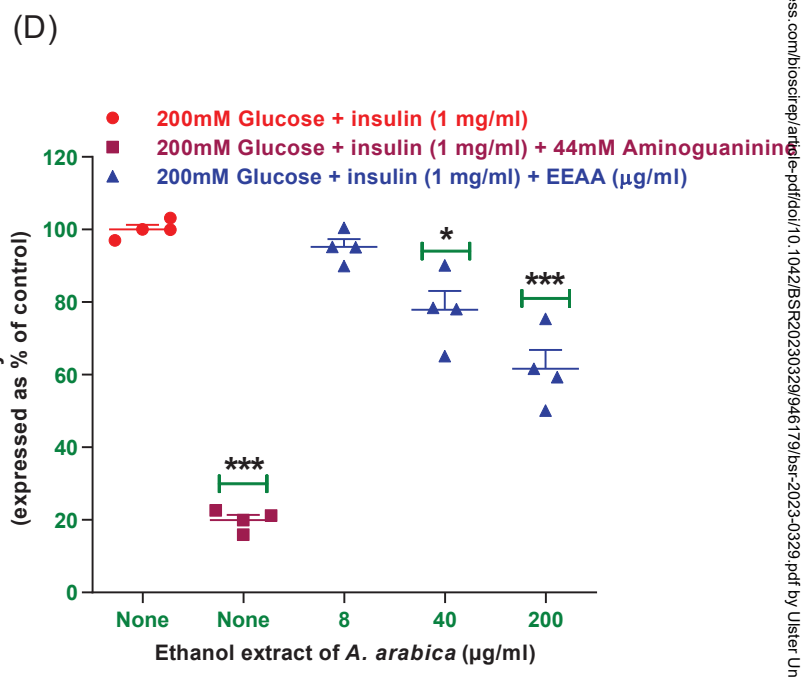
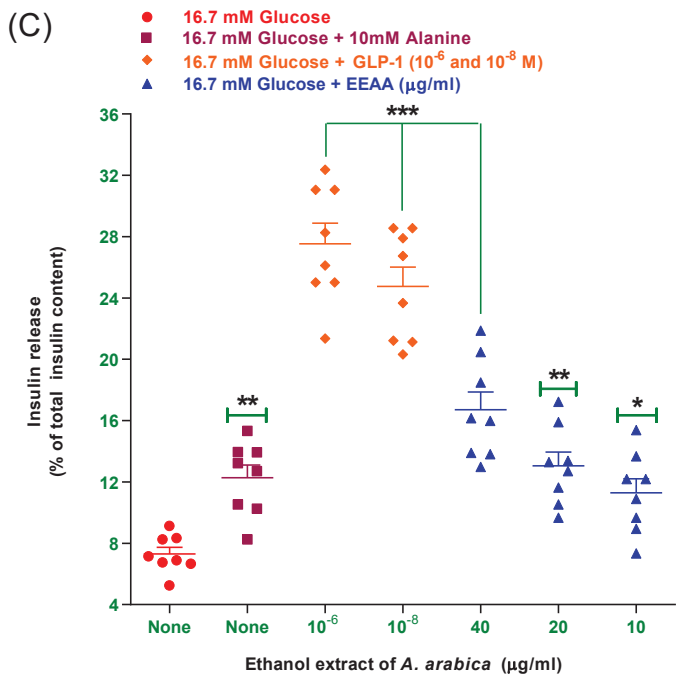
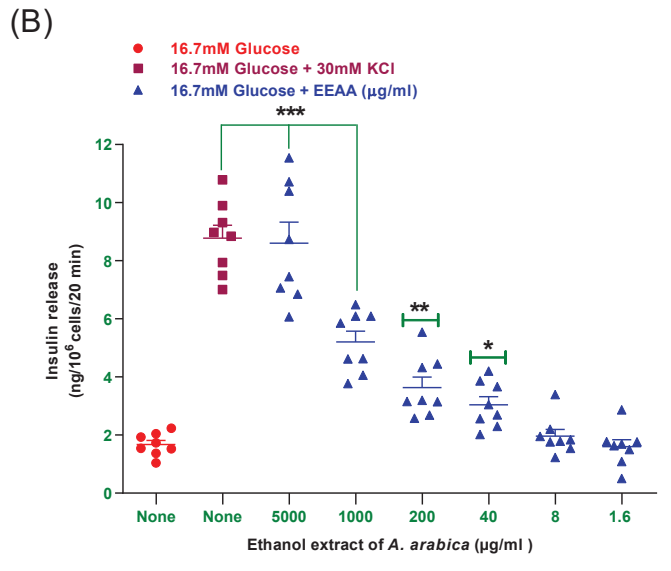
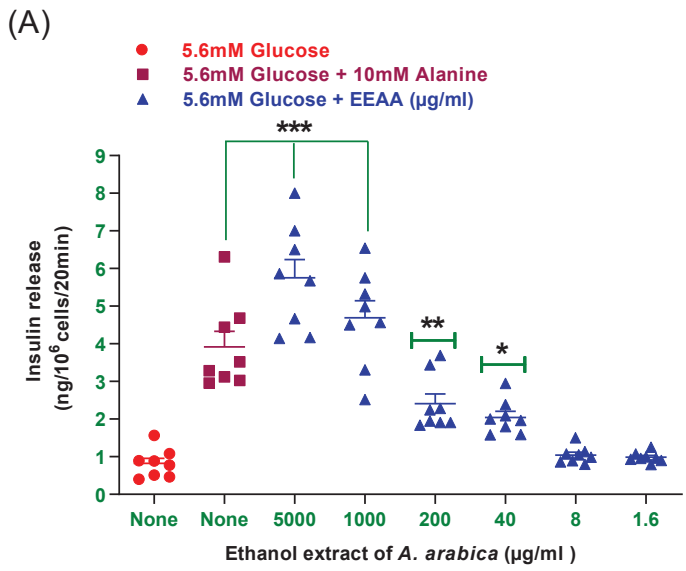
783 Values are mean \pm SEM; $n = 4-8$ for insulin secretion and glycation of protein. *, **, *** $p <$
784 $0.05-0.001$ compared to control. ϕ $p < 0.05$ and $\phi\phi\phi$ $p < 0.001$ compared to 5.6 mM glucose
785 with EEAA. Δ , $\Delta\Delta$, $\Delta\Delta\Delta$ $p < 0.05-0.001$ compared to respective incubation without EEAA.
786 EEAA, ethanol extract of *Acacia arabica* (bark).

787 **Figure 2: Effects of ethanol extract of *Acacia arabica* (EEAA) bark on (A) membrane**
788 **potential and (B) intracellular calcium in clonal pancreatic BRIN-BD11 β -cells and, (C-**
789 **G) glucose uptake by differentiated 3T3L1 adipocytes, (H) starch digestion and (I)**
790 **glucose diffusion *in vitro*.**

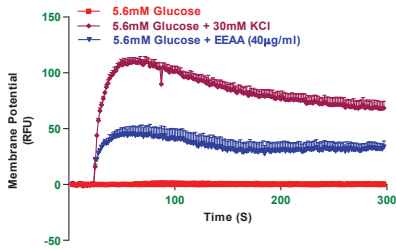
791 The intensity of fluorescence was measured for cells incubated with EEAA (E) minus or (F)
792 plus 100 nM insulin. The images were captured at 10x magnification. (G) Glucose uptake in
793 3T3L1 cells and percentage of glucose liberation from (H) starch digestion and (I) glucose
794 diffusion *in vitro* were represented in the scatter dot plot. The Values are mean \pm SEM; $n = 6$
795 for membrane potential and intracellular calcium, $n = 4$ for glucose uptake, starch digestion
796 and glucose diffusion. *, **, *** $p < 0.05-0.001$ compared to control.

797 **Figure 3: Effects of ethanol extract of *Acacia arabica* (EEAA) bark on (A) *in vitro***
798 **dipeptidyl peptidase-4 (DPP-IV) enzyme, (B) glucose tolerance, (C) plasma insulin, (D)**
799 **plasma DPP-IV and (E) active GLP-1 (7-36) in HFF rats.**

800 Parameters were measured before and after oral administration of glucose alone (18 mmol/kg
801 body weight, control) or with EEAA (250 mg/5 ml/kg body weight), sitagliptin and
802 vidagliptin (both at 10 μ mol/5 ml/kg, body weight) *in vivo*. Plasma active GLP-1 (7-36)
803 levels were evaluated at 60 min following treatment. Values are mean \pm SEM; $n = 4$ for *in*
804 *vitro* DPP-IV enzyme activity and $n = 6$, for *in vivo* parameters. *, **, *** $p < 0.05-0.001$
805 compared to control and Δ , $\Delta\Delta$, $\Delta\Delta\Delta$ $p < 0.05-0.001$ compared to high-fat-fed diet control rats.



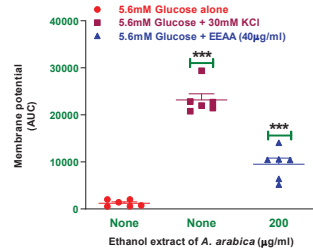
(A)



(C)

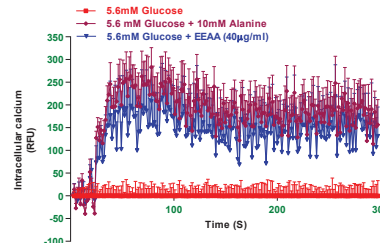
Control

(B)



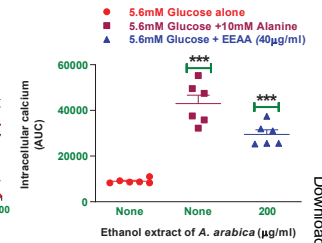
(D)

100nM Insulin



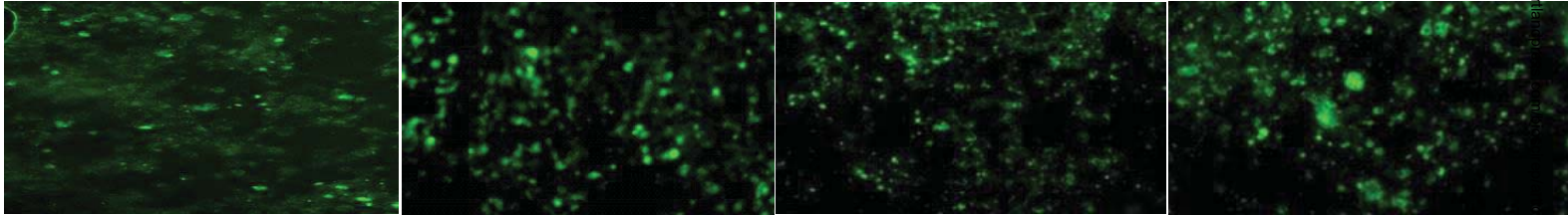
(E)

EEAA (40 µg/ml)

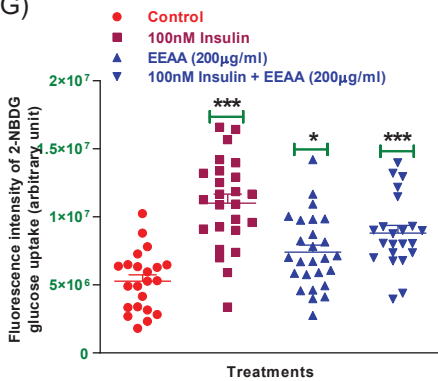


(F)

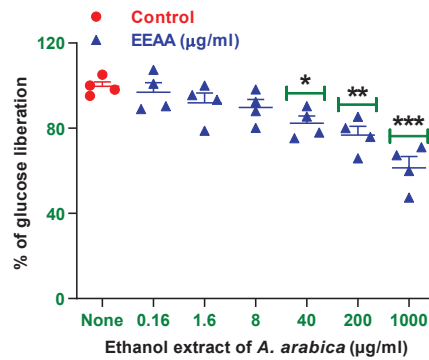
100nM Insulin + EEAA (40 µg/ml)



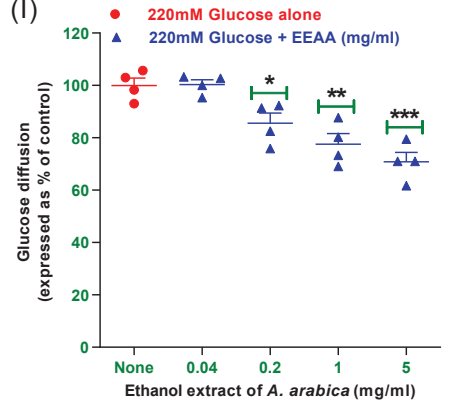
(G)



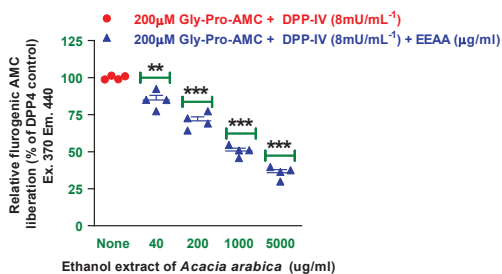
(H)



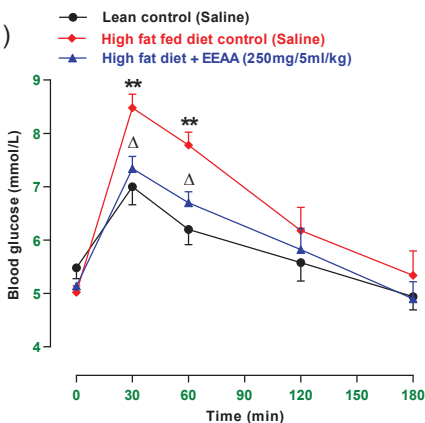
(I)



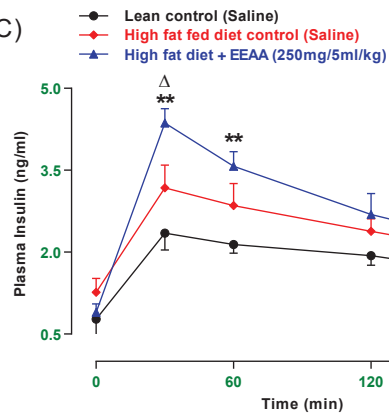
(A)



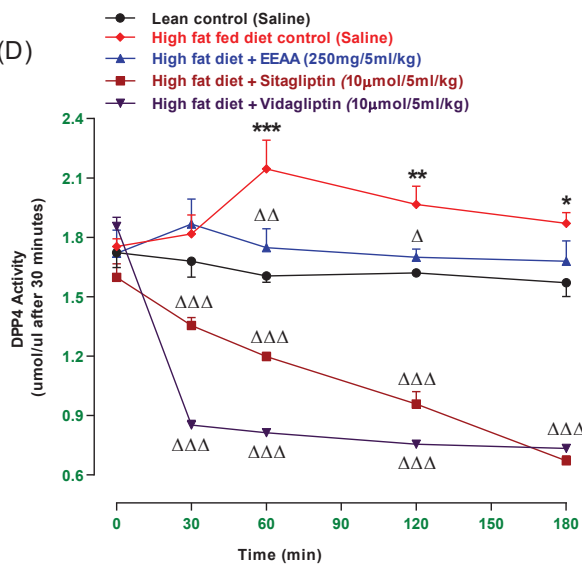
(B)



(C)



(D)



(E)

