Evaluation of the glycemic effect of *Ceratonia siliqua* pods (Carob) on nicotinamide-streptozotocin-induced diabetic rats model

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Background. *Ceratonia siliqua* pods (carob) has been nominated to control high blood glucose of diabetics. In Yemen, however, its antihyperglycemic activity has not been yet assessed. Thus, this study evaluated the *in vitro* inhibitory effect of the methanolic extract of carob pods against α-amylase and α-glucosidase and the *in vivo* glycemic effect of such extract in nicotinamide-streptozotocin-induced diabetic rats.

Methods. DPPH and FRAP were applied to evaluate the antioxidant activity of carob**.** *In vitro* cytotoxicity of carob was conducted on human hepatocytes (WRL68) and rat pancreatic β-cells (RIN-5F). Acute oral toxicity of was conducted on a total of 18 male and 18 female *Sprague-Dawley* (SD) rats, which were subdivided into three groups (n=6), namely; high and low dose carob-treated (CS5000 and CS2000, respectively) as well as normal control (NC) receiving a single oral dose of 5000 mg kg^{-1} carob, 2000 mg kg^{-1} carob and 5 ml kg^{-1} distilled water for 14 days, respectively. Alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, total bilirubin, creatinine and urea were assessed. Livers and kidneys were harvested for histopathology. *In vitro* inhibitory effect against α-amylase and α-glucosidase was evaluated. *In vivo* glycemic activity was conducted in 24 male SD rats which were previously intraperitoneally injected with 55 mg kg^{-1} streptozotocin (STZ) followed by 210 mg kg^{-1} nicotinamide to induce type 2 diabetes mellitus. An extra non-injected group (n=6) was added as a normal control (NC). The injected-rats were divided into four groups (n=6), namely; diabetic control (D0), 5 mg kg^{-1} glibenclamide-treated diabetic (GD), 500 mg kg^{-1} carob-treated diabetic (CS500) and 1000 mg kg^{-1} carob-treated diabetic (CS1000). All groups received a single oral daily dose of their treatment for four weeks. Body weight, fasting blood glucose (FBG), oral glucose tolerance test, biochemistry, insulin and hemostatic model assessment were assessed. Pancreas was harvested for histopathology.

Results. Carob demonstrated a FRAP value of 3191.67 ± 54.34 μmoL Fe^{++} and IC_{50} of DPPH of 11.23 ±0.47 μg mL^{-1}. *In vitro,* carob was non-toxic on hepatocytes and pancreatic β-cells. In acute oral toxicity, liver and kidney functions and their histological sections showed no abnormalities. Carob exerted *an in vitro* inhibitory effect against α-amylase and α-glucosidase with IC_{50} of 92.99±0.22 and 97.13±4.11 μg mL^{-1}, respectively. In diabetic induced rats, FBG of CS1000 was significantly less than diabetic control. Histological pancreatic sections of CS1000 showed less destruction of β-cells than CS500 and diabetic control.

Conclusion. Carob pod did not cause acute systemic toxicity and showed *in vitro* antioxidant effects. On the other hand, inhibiting α-amylase and α-glucosidase was evident. Interestingly, high dose of carob exhibits an *in vivo* antihyperglycemic activity and warrants further in depth study to identify the potential carob extract composition.

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2 **Nicotinamide-Streptozotocin-Induced Diabetic Rats Model**

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17 **Abstract**

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20 study evaluated the *in vitro* inhibitory effect of the methanolic extract of carob pods against α-

21 amylase and α-glucosidase and the *in vivo* glycemic effect of such extract in nicotinamide-

22 streptozotocin-induced diabetic rats.

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26 *Dawley* (SD) rats, which were subdivided into three groups (n=6), namely; high and low dose

27 carob-treated (CS5000 and CS2000, respectively) as well as normal control (NC) receiving a

28 single oral dose of 5000 mg kg^{-1} carob, 2000 mg kg^{-1} carob and 5 ml kg^{-1} distilled water for 14

29 days, respectively. Alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase,

30 total bilirubin, creatinine and urea were assessed. Livers and kidneys were harvested for

31 histopathology. *In vitro* inhibitory effect against α-amylase and α-glucosidase was evaluated. *In*

32 *vivo* glycemic activity was conducted in 24 male SD rats which were previously intraperitoneally

33 injected with 55 mg kg^{-1} streptozotocin (STZ) followed by 210 mg kg^{-1} nicotinamide to induce

34 type 2 diabetes mellitus. An extra non-injected group (n=6) was added as a normal control (NC).

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36 glibenclamide-treated diabetic (GD), 500 mg kg^{-1} carob-treated diabetic (CS500) and 1000 mg kg^{-1}

37 carob-treated diabetic (CS1000). All groups received a single oral daily dose of their treatment for

38 four weeks. Body weight, fasting blood glucose (FBG), oral glucose tolerance test, biochemistry,

39 insulin and hemostatic model assessment were assessed. Pancreas was harvested for

40 histopathology.

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50 dose of carob exhibits an *in vivo* antihyperglycemic activity and warrants further in depth study to

51 identify the potential carob extract composition.

INTRODUCTION

Diabetes mellitus (DM) is a widespread metabolic disorder that is estimated to affect 640 million

by 2040 (International Diabetes Federation 2016). DM occurs owing to a defect in either insulin

secretion, insulin action, or both leading to an increase in blood glucose (Gamboa-Gómez et al.

2017). Accordingly, DM is classified into either type 1 diabetes mellitus (T1DM) which is insulin

dependent or type 2 diabetes mellitus (T2DM) which is non-insulin dependent (WHO 2016).

However, T2DM is the most common type of DM constituting 90- 95% of all cases of DM

worldwide (Ahmed et al. 2016; Tripathi 2013).

T2DM is mainly managed with oral hypoglycemic agents. However, due to the potential side

effects of those agents, World Health Organization (WHO) recommends using alternative

medicines (e.g. natural products) (Abdel-Sattar et al. 2013) as a second choice (AM Saghir et al.

2016). As a matter of fact, more than 1200 species of medicinal plants could be used as an

antidiabetic (Abdel-Sattar et al. 2013; Alarcon-Aguilar et al. 2002; Hernandez-Galicia et al. 2002).

Carob (*Ceratonia siliqua* L) is an evergreen plant (Roseiro et al. 2013), which was reported as a

native plant to Yemen (Battle & Tous 1997). Traditionally, *Ceratonia Siliqua* (carob) is used as

an antitussive, antidiarrheal and a diuretic (Baytop 1984; Gulay et al. 2012; Merzouki et al. 1997).

However, *Ceratonia Siliqua* pods (Carob) were reported to exert several pharmacological

properties such as antioxidant, anti-ulcer and anti-inflammatory (Kivcak et al. 2002; Rtibi et al.

2015a; Rtibi et al. 2017a; Rtibi et al. 2016).

In the folk Yemeni medicine, diabetics claim that carob pods could reduce their elevated blood

sugar. However, no study was conducted to explore the glycemic effect of the Yemeni cultivar of

carob pods. Consequently, this study sought to evaluate the glycemic and the antioxidant activities

of the methanolic extract of carob in nicotinamide-streptozotocin-induced diabetic rat's model.

MATERIALS AND METHODS

Materials and chemicals

Streptozotocin (Merck Millipore, USA), nicotinamide, sodium citrate monohydrate, phosphate-buffered saline (PBS) and Glucose anhydrous (Sigma Aldrich, USA). Citric acid (Merck, USA), glibenclamide (Sigma Aldrich, USA), formaldehyde (HmbG chemicals, Germany), One-touch glucometer (Accu-Chek Performa, Roche, Mannheim, Germany). Rat Insulin ELISA Kit (ER1113, Wuhan Fine Biological Technology Co., Ltd., China). 2,4,6-Tri (2- pyridyl)-s-triazine, Folin-Ciocalteu reagent and ferric chloride (Sigma Aldrich, USA). Cellulose extraction thimble (Tokyo Roshi Kaisha, Ltd, Tokyo, Japan (Sigma Aldrich, USA), Ferrous sulphate heptahydrate (Essex, UK). Amylase and glucosidase enzymes, 3, 5-dinitrosalicylic acid (DNSA) and 4-Nitrophenyl ($_p$-D-glucopyranoside ($_p$-NPG) substrate, monobasic potassium phosphate and potassium tartrate tetrahydrate (Sigma Aldrich, USA), α-acarbose 95 % (Acros organic, USA). Potato starch (Sigma Aldrich, USA).

Collection of plant material

Totally, two kilograms of unripe pods of carob were collected from Bani Yousef region, 70 km from Taiz governorate, Yemen. The pods were collected at 8 am, 15th May 2014. The shoots were wrapped in aluminum foil sheets, put in ice-filled box to isolate it from sunlight and external temperature. The unripe carob samples were transported directly to the Laboratory of Pharmacognosy Department, Faculty of Pharmacy, Sana'a University, Yemen. The plant was verified and authenticated by a taxonomist and given a voucher specimen No. (CSL/2014/8/1), which was deposited at the Laboratory of Pharmacognosy, Faculty of Pharmacy, Sana'a University, Yemen.

99   **Extraction**

100   The unripe carob pods were washed with purified water to eliminate debris and dried with a fan.

101   Then, the pods were chopped to small pieces and left to dry at room temperature (28.0±2°C) for

102   three weeks. Next, chopped pieces were ground with a grinder to get fine powder at department of

103   Pharmacy, Faculty of Medicine, University of Malaya. One kilogram of carob powder was

104   extracted using Soxhlet method as previously described with some modification (Jadhav et al.

105   2009) Extraction process was performed by methanol using Soxhlet extractor under 50 °C. Filter

106   paper (Whatman No. 1) was used to filter the liquid extract. Rotavapor evaporator was used to

107   separate the solutes from the solvents at 40 °C and the filtrate was allowed to dry in dark place.

108   Finally, the dried filtered extract was kept under -20 °C for later work.

109   *In vivo* **and** *in vitro* **experimental model design of study**

110   Figure 1 Flow chart of the experimental design

111   **Quantification of total phenolic and flavonoid content**

112   Total phenolic content (TPC) of the methanolic extract of carob was determined using Folin-

113   Ciocalteu assay (Chan et al. 2009). Gallic acid was used as reference. Totally, 20 μL of the extract

114   and gallic acid (1 mg mL^{-1}) were pipetted into 96-well microplate followed by addition of 50 μl of

115   10% Folin-Ciocalteu reagent to be incubated for 3.0 minutes. Then 100 μL of sodium carbonate

116   (10%) was added followed by incubation for one hour. The absorbance was measured at 765 nm

117   spectrophotometrically (Infinite M 200 Tecan, Switzerland). The phenolic content was represented

118   as mg of gallic acid equivalent/g extract.

119   Total flavonoid content (TFC) was estimated using $AlCl_3$ method (Nabavi et al. 2008) in which 10

120   μL of the extract and quercetin were transferred into 96-well plate, followed by addition of 60 μl

121   of DMSO. Then, 10 μl of 10% (w/v) aluminium chloride, 10 μl of 1 M potassium acetate and 120

122 μl of deionized water were added to the mixture, which was incubated for 30 minutes at room

123 temperature. Serial dilutions of quercetin (500-31.25 μg mL^{-1}) were prepared to generate the

124 calibration curve. The absorbance was measured at 415 nm spectrophotometrically (Infinite M 200

125 Tecan, Switzerland). The flavonoid content was presented as mg quercetin equivalent/g extract.

126 **Evaluation of antioxidant properties of carob methanolic extract**

127 **Radical-scavenging activity of carob against free radicals of DPPH**

128 Radical scavenging activity of the methanolic extract of carob was determined using the stable

129 free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) with slight modifications (Alshawsh et al.

130 2012). Briefly, 1.2 mg of DPPH was thawed out in 30 mL of DMSO to prepare 100 μM DPPH

131 reagent solution. Stock sample (1.0 mg/1.0 mL DMSO) was diluted. Serial dilutions; 500, 250,

132 125, 62.50, 31.25, 15.62 and 7.18 μg mL^{-1}of stock sample were prepared. Totally, 25 μL of sample

133 and 150 μL of DPPH solution were transferred into 96-wells plate. Next, the mixture was incubated

134 in a dark place at room temperature for 25 minutes. The absorption was measured

135 spectrophotometrically (Infinite M 200 Tecan, Switzerland) at 517 nm. Quercetin was used as a

136 positive control. The amount of the extract was replaced by DMSO in case of negative control.

137 Percentage inhibition was obtained using the following equation:

138 % Inhibition= [(Ac-As)/Ac] *100.

139 Where Ac is the absorbance of negative control, As is the absorbance of test sample.

140 The concentration of the methanolic carob extract that was able to inhibit 50% of the free radicals

141 of DPPH (IC$_{50}$) was obtained from the calibration curve of sample and positive control using a non-

142 linear regression analysis and the results were expressed as an IC$_{50}$ value ± SD. All determinations

143 were conducted in triplicate to evaluate the IC$_{50}$.

144 **Ferric reducing antioxidant power assay (FRAP)**

145 Measuring of ferric reducing antioxidant activity of the methanolic extract was performed

146 according to (Benzie & Strain 1996). Working FRAP reagent was prepared through mixing 300

147 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCL, and 20

148 mM ferric chloride ($FeCl_3.6H_2O$). Calibration curve was plotted using $FeSO_4 \cdot 7H_2O$ serial

149 concentrations between 100-1000 mg mL^{-1}. Freshly prepared FRAP working reagent was warmed

150 at 37°C for 5 min, then 30 µl of the extract, Trolox and quercetin (1.0 mg dissolved in 1.0 mL

151 DMSO) were transferred into 96-well microplate and mixed with 300 µL of FRAP reagent. Then,

152 absorbance was measured at 593 nm against the blank (DMSO). The values were represented as

153 µmoles of ferrous sulphate equivalent/mg extract.

154 ***In vitro* inhibitory effect of carob against α-amylase and α-glucosidase**

155 The α-amylase inhibition capacity of the extract was determined according to Loizzo et al. (2007).

156 In brief, 20 µL of each test sample (1 mg mL^{-1}extract) dissolved in DMSO or standard Acarbose

157 95 % (Acros organic, USA) and 50 µL 2U mL^{-1} of α-amylase enzyme (Porcine Pancreas Amylase;

158 2mg/10 mL; 10 Units/mg; Sigma-Aldrich, USA) dissolved in cold deionized water were added

159 into each well. After incubation for 10 min at 28 °C, 100 µL of starch solution (0.5 % w/v (Sigma-

160 Aldrich, USA) prepared by mixing 250 mg potato starch in previously prepared 50 mL phosphate

161 buffer pH 6.9 {prepared by mixing 20 mM monobasic sodium phosphate and 6.7 mM of sodium

162 chloride} and heated at 60 °C for 15 min) was added to the reacting mixture. Thereafter, the

163 reaction mixture was re-incubated for 10 min at 28°C and 100 µL of colorimetric DNSA reagent

164 96 mM (Prepared by mixing 12 g of sodium potassium tartrate tetrahydrate in 8 mL of 2 M NaOH

165 and 0.5 mg 3,5-dinitrosalicylic acid solution) was added with mixing the contents well. The

166 reaction was terminated by incubating the mixture in a water bath 86 °C for 15 min and later was

167   cooled to room temperature. The blank was conducted in a similar method, with the replaced α-

168   amylase enzyme by deionized water. All the reagents involved the α-amylase enzyme with the

169   exception of the test extracts was pointed as a reference sample. The absorbance of reaction was

170   measured by using UV-Visible spectrophotometry (Infinite M 200 Tecan, Switzerland) at 540nm

171   and the α-amylase inhibitory activity was expressed as percentage inhibition. The assay was

172   carried out in 96-well microplates (Solid clear F-bottom, Greiner Bio One, Austria). The

173   percentage of inhibition was obtained using the following formula: % inhibition = [A reference –

174   (A sample – A blank)/ A reference)] ×100.

175   Regarding α-glucosidase inhibitory activity of carob methanolic extract, it was assessed

176   spectrophotometrically according to Oboh et al. (2012) with few modifications. Briefly, 100 μL

177   phosphate buffer solution of 2.5 U mL^{-1} α-glucosidase enzyme (Saccharomyces cerevisiae; 2.5mg

178   10 mL^{-1}; 10 U mg^{-1}; Sigma-Aldrich. St Louis, USA) and 50 μL of each test sample solution (1 mg

179   mL^{-1} extract) dissolved in 30 % DMSO were added in each well and incubated at 25 °C for 10 min.

180   Then, 50 μL of 4-Nitrophenyl (3-D-glucopyranoside ($_P$-NPG) substrate solution (5 mM of $_P$-NPG

181   (Sigma-Aldrich, St Louis, USA) 7.53mg/5mL dissolved in pH 6.9 phosphate buffer which

182   prepared by mixing with 100mM monobasic potassium phosphate); was added to the reacting

183   mixture and re-incubated for 5 min at 25 °C. Thereafter, 80 μL of 0.1M sodium carbonate solution

184   was added to terminate the catalytic reaction. Acarbose 95 % (Acros organic, USA) dissolved in

185   30% DMSO was used as a positive control. The reference sample contained the same reaction

186   mixture except the same amount of test sample solution and positive control that were replaced by

187   phosphate buffer solution. The blank was included all reagents and test samples with the exception

188   of α-glucosidase enzyme. All measurements were performed in triplicate. The absorbance of the

189   reaction was recorded on UV-Visible spectrophotometry (Infinite M 200 Tecan, Switzerland) at

190   405 nm. The assay was carried out in 96-well microplates (Solid clear F-bottom, Greiner Bio One,

191   Austria). The inhibitory rate of samples on α-glucosidase enzyme was obtained using the following

192   formula: % inhibition = [A reference– (A sample–A blank)/ A reference)] ×100.

193   ***In vitro* cytotoxicity of the methanolic extract of carob**

194   **Cell culture**

195   RIN-5F (rat pancreatic β-cell line) and WRL 68 (human hepatic cell line) (ATCC; Manassas, VA,

196   USA) were maintained in RPMI-1640 and DMEM medium, respectively. 10% of fetal bovine

197   serum (FBS) and 1% antibiotics (penicillin-streptomycin) were supplemented to the media, then

198   it was incubated under an atmosphere of 5% CO_2 and 95% humidified air at 37°C. The medium

199   was replaced twice weekly until confluent cell monolayer was formed and observed under an

200   inverted microscope.

201   **Cellular viability assay**

202   The inhibitory effect of methanolic extract of carob was determined by MTT assay. The reduction

203   of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) by the mitochondrial

204   dehydrogenase in cells and producing purple formazan is the principle of the experiment. In which

205   5×10^3 of WRL 68 and RIN-5F per well were seeded in triplicate in 96-well microplates and

206   incubated for 24 h to be attached at 37 °C with 5% CO_2 saturation. After 24 h of incubation,

207   decreasing concentrations of carob methanolic extract (such as 1000, 500, 250, 125, 60, 30, 15 and

208   7μmol) were prepared (0.025% DMSO) and transferred to the cells with incubating for 24, 48,72

209   h at 37 °C and 5% CO_2. Subsequently, 20 μL of MTT solution (5 mg mL^{-1}) was added to the

210   treated cells in a dark place, covered with aluminum foil and incubated for 4 h. All media was

211   discharged and a total of 100 μL of DMSO was poured into each well until the purple formazan

212   crystals dissolved (Jayash et al. 2016; Jayash et al. 2017). The blank was performed in a similar

213   manner but the test samples were replaced by media (0.025% DMSO). The plates were measured

214   using a microplate reader UV-Visible spectrophotometry (Infinite M 200 Tecan, Switzerland) at

215   absorbance 570 nm. The tests were carried out in 96-well microplates (Solid clear F-bottom,

216   Greiner Bio One, Austria). All experiments were conducted in triplicate to evaluate the IC_{50} (The

217   concentration of the methanolic carob extract that was able to inhibit 50% of cells viability). The

218   percentage of cell viability was calculated using the following equation: Cell viability%= A treated

219   cells/ A blank ×100.

220   According to Jayash et al. (2017), the cytotoxic effect of each serial dilution of the methanolic

221   extract of carob was evaluated.

222   **Acute oral toxicity of the methanolic extract of carob**

223   Acute oral toxicity was conducted according to the guidelines of OECD (OECD. 2005) and ethical

224   approval **(2014-07-01/PHARM/MAQA)** that was issued by the Institutional Ethics Committee,

225   University of Malaya, Malaysia. The animals were kept under the standard conditions of housing

226   as mentioned above. Totally, 36 adult SD-rats (18 males and 18 females) were randomly dispersed

227   into normal control (n=6), low dose methanolic carob extract (CS2000) (n=6) and high dose

228   methanolic carob extract (CS5000) (n=6), which received an oral single dose of 5 mL kg^{-1} normal

229   saline, 2000 mg kg^{-1} carob and 5000 mg kg^{-1} carob during the 14 days of the acute oral toxicity

230   time course, respectively. Experimental rats were fasted overnight before dosing. Immediately

231   after dosing, rats were observed at half an hour, 2, 4, 24 and 48 hours for any toxicological signs.

232   By the 14th day, rats were fasted overnight, anaesthetized, scarified to collect blood samples via

233   cardiac puncture. Blood samples were centrifuged at 1500 rpm to obtain serum to measure liver

234   function tests, kidney function tests and total protein. as well as liver and kidney were harvested

235   and their sections were processed to be stained with H&E stain for histopathology evaluation.

236   *In vivo* **glycaemic activity of the methanolic extract of carob**

237   **Experimental animals**

238   Totally, 30 male *Sprague–Dawley* rats (7-8 weeks) were obtained from the Animal House Unit

239   (AEU) in the Faculty of Medicine, University of Malaya. Malaysia. Animals were kept following

240   the Guide for the Care and Use of Laboratory Animals published by (Institute for Laboratory

241   Animal Research 2010). The use of animals and the study was conducted according to the ethic

242   approval issued by Institutional Animal Care and Use Committee (FOM IACUC), Faculty of

243   Medicine, University of Malay, Malaysia in 7 June 2014 under ethic number **(2014-07-**

244   **01/PHARM/MAQA)**. The animals were kept within the AEU at Faculty of Medicine, University

245   of Malaya and allowed to acclimatize for one week under 20 ± 3 ºC, relative humidity (30–70%)

246   and 12 h light/dark cycle with free access to food and water.

247   **Induction of type 2 diabetic animal model**

248   According to the protocol of (Arya et al. 2012a) , the rats were fasted overnight and injected with

249   a single intraperitoneal dose of 55 mg kg-1 of freshly prepared streptozotocin (STZ) in 0.1 M

250   citrate buffer (pH 4.5). Immediately after 15 mins, rats were injected intraperitoneally with 210

251   mg kg^{-1} nicotinamide (NAD) which exert an antioxidant capacity that minimize the cytotoxic

252   actions of STZ and protects pancreatic β-cell against the destructive effect of STZ (Furman 2015).

253   After 96 hours, rats were fasted overnight except free access for tap water to measure fasting blood

254   sugar using Accu-Chek glucometer. The injected rats were monitored for 10 days and those which

255   attained fasting blood sugar between 10-14 mmol L^{-1} were considered type 2 diabetic.

Dose rationale of carob

In this study, the doses of the methanolic carob extract were selected to be 500 mg kg^{-1} as a low dose (CS500) and 1000 mg kg^{-1} as a high dose (CS1000) according to the pervious study by Rtibi et al. (2015b).

The experimental design

The type2 induced rats were randomly assigned into four groups (n=6); namely, diabetic untreated control (D_0), diabetic glibenclamide-treated (GD), diabetic low dose carob-treated (CS500) and diabetic high dose carob-treated (CS1000), which received a single oral daily dose of 5 mL kg^{-1} normal saline, 5 mg kg^{-1} glibenclamide (Hafizur et al. 2015), 500 mg kg^{-1} carob and 1000 mg kg^{-1} carob for four weeks respectively. An extra untreated non-diabetic control (NC) (n=6) was added and given oral daily single dose of 5 mL kg^{-1} of normal saline for four weeks. Blood glucose level was monitored weekly from the tail vein using Accu-Chek glucometer. Body weight was recorded at the day of starting (initial body weight) and cession of treatment (final body weight). By the 28th days of treatment, rats were anaesthetized using ketamine (50 mg kg^{-1}) and xylazine (5.0 mg kg^{-1}). Then, 5 mL of blood samples were collected for blood analysis through cardiac puncture. Blood samples were centrifuged at 2000 rpm for 10 min and serum samples were separated and stored at -80 for the future use. Subsequently, rats were scarified by increasing anesthetic dose (ketamine 150 mg kg^{-1} & xylazine 15.0 mg kg^{-1}) to obtain pancreas for gross pathology examination.

Oral glucose tolerance test

Oral glucose tolerance test (OGTT) evaluated the glucose challenge ability of each animal one week prior to animal sacrifice (Arya et al. 2012b). After fasting rats overnight, tip tail fasting blood glucose was measured at 0 time. Twenty minutes after rats had received their own

278   treatment, rats were given a single oral dose of glucose solution (3 g/kg), and blood glucose was

279   measured at 30, 60, 90 and 120 minutes.

280   **Evaluation of insulin resistance**

281   After measuring fasting serum insulin, homeostatic model assessment (HOMA-IR, HOMA-β

282   and HOMA-S) was estimated (Mather 2009). Serum insulin was quantified by the enzyme-

283   linked immunosorbent assay (ELISA) using an ultrasensitive rat insulin ELISA kit (ER1113,

284   Wuhan Fine Biological Technology Co., Ltd., China) and read at 450 nm with a microplate

285   reader (Hydroflex Elisa, Chemo pharm, Austria) according to the manufacturer's instructions.

286   HOMA-IR, HOMA-β and HOMA-S were estimated in term of fasting insulin ($\mu U\ mL^{-1}$) and

287   fasting blood glucose ($mmol\ L^{-1}$) using HOMA 2 calculator software (Diabetes Trial Unit 2004).

288   **Histopathological examinations**

289   For histological analysis, pancreatic tissues were collected directly after sacrificing animals and

290   fixed with formaldehyde 10% (v/v). Histopathological examinations were accomplished by an

291   experienced pathologist. The pancreases sections were stained with hematoxylin and eosin (H&E).

292   Histological features including morphological configuration, size of islets and number of β-cells

293   were examined and differentiated for each group.

294   **Statistical analysis**

295   The statistical analysis was determined using a statistical software package (SPSS for Windows,

296   version 23, IBM Corporation, NY, USA) and analysis of variance (*ANOVA*), followed by Tukey's-

297   SHD multiple range post-hoc test. The IC_{50} was calculated from the linear regression analysis.

298   Pearson correlation coefficient analysis was done to evaluate the correlation between phenolic and

299   flavonoid contents against antioxidant activities. Values were considered statistically significant

300   at $P < 0.05$. Results were expressed as either means ± SD or means ± SE.

301 **Results**

302 **Antioxidant activity**

303 Table 1 demonstrated that the phenolic content (y = 0.0075x - 0.0123, R^2 = 0.9817) was 127.02 ±

304 7.18 mg GAE g^{-1}, while the flavonoid content (y = 0.0023x - 0.0165, R^2 = 0.9979) was 49.74 ±

305 0.88 mg QE g^{-1}, which means that flavonoid content approximately accounted for 39.2% of the

306 total phenolic content. According to IC_{50} values, the scavenging activity of the methanolic extract

307 against the free radicals of DPPH (y = 13.339 ln (x) + 17.685, R^2 = 0.9727) was close to that of

308 Trolox, but it was double that of quercetin. Similarly, FRAP value (y= 0.0002x + 0.002, R^2

309 =0.9649) of the methanolic extract was approximately half that of either quercetin or Trolox.

310 Pearson correlations analysis displayed that the total phenolic content of carob methanolic extract

311 had a strong positive correlation with FARP assay (r= 0.918, $P<0.01$) and a strong negative with

312 DPPH radical (r= -0.910, $P<0.01$).

313 Likewise, total flavonoid content of carob methanolic extract reflects a strong positive correlation

314 with FRAP assay (r= 0.886, $P<0.01$) and a strong negative correlation with DPPH radical (r= -

315 0.867, $P<0.01$), (Table 2).

316 **Toxicity assessment of the methanolic extract of carob**

317 *In vitro* **cytotoxicity**

318 In the current study, the toxic effect of the methanolic extract of carob against RIN-5F (pancreatic

319 β-cells) and WRL 68 (human hepatocytes cells) was assessed. The percentage of viability of either

320 RIN-5F (Fig.2) or WRL 68 (Fig.3) was upper than 30% after 24, 48 and 72 hours of exposure to

321 the different serial concentrations of the methanolic extract of carob.

322 *In vivo* **acute oral toxicity**

323 Neither morbidity nor mortality in male or female rats was observed. Similarly, no abnormal signs or

324 behavioral changes were observed during the course of acute oral toxicity. Table 3 showed that no

325 significant difference was observed in body weight of male and female rats. Like body weight, absolute

326 liver, kidney, heart and pancreas weights, as well as relative liver, kidney, heart and pancreas weights

327 of male and female rats were non-significantly different. Table 4 showed that only alkaline phosphatase

328 and urea of high dose carob-treated (CS5000) were significantly increased as compared to normal

329 control (NC) ($P \leq 0.05$ and $P \leq 0.01$, respectively). In female rats, only serum creatinine showed

330 significant increased as compared to normal control ($P \leq 0.01$).

331 **Histopathology of acute oral toxicity**

332 The liver histological sections of normal control (G and J), low dose carob-treated CS2000 (H and

333 K) and high dose carob-treated CS5000 (I and L) of both male and female were of normal

334 architecture of hepatocytes at the level of their cell membrane, nuclei or even cytoplasm. In

335 addition, no congestion in portal veins, no necrotic lesions, no inflammatory signs or fatty

336 infiltrations could be observed. Similarly, kidney histological sections of normal control (A and D),

337 CS2000 (B and E) and CS5000 (C and F) of both male and female were of normal architecture of

338 tubules, Bowman's capsule, Malpighian corpuscles (Fig.4).

339   **Glycemic effect of the methanolic extract of carob**

340   *In vitro* **glycemic effect**

341   The IC_{50} of carob methanolic extract (92.99 ± 0.22 µg mL^{-1}) was higher than that of α-acarbose

342   (23.33 ± 0.73 µg mL^{-1}) against α-amylase (Fig.5). Similarly, the IC_{50} of the methanolic extract

343   (97.13 ± 4.11 µg mL^{-1}) against α-glucosidase was higher than that of α-acarbose (27.05 ± 0.99 µg

344   mL^{-1}) (Fig.6).

345   *In vivo* **glycemic effect**

346   **Body weights as well as absolute and relative organs weight of STZ-NAD diabetic animal**

347   **model**

348   Table 5 demonstrates that body weight of low dose carob-treated (CS500) and high dose carob-

349   treated (CS1000) was significantly reduced more than untreated type 2 diabetic rats (D0) ($P \le 0.05$

350   and $P \le 0.01$, respectively). On the other hand, absolute pancreas weight (APW) and relative

351   pancreas weight (RPW) of CS1000 were significantly lower than that of untreated type 2 diabetic

352   rats group ($P \le 0.001$ and $P \le 0.01$, respectively).

353   **Weekly fasting blood sugar of STZ-NAD diabetic animal model**

354   Figure 7 showed that normal control (NC) showed a higher significant fasting blood glucose (FBG)

355   than untreated type 2 diabetic rats (D0) at the baseline as well as the end of the 1st, 2nd, 3rd and 4th

356   week of treatment ($P \le 0.001$, $P \le 0.001$, $P \le 0.001$, $P \le 0.001$ and $P \le 0.001$, respectively). In

357   addition, FBG of glibenclamide-treated type 2 diabetic (GD) was significantly lower than

358   untreated type 2 diabetic rats at the end of the 2nd, 3rd, and 4th weeks ($P \le 0.001$, $P \le 0.001$ and P

359   ≤ 0.001, respectively). Moreover, low dose carob-treated (CS500) showed a lower significant FBG

360   than untreated type 2 diabetic rats only at the end of 4th week ($P \le 0.001$) while high dose carob-

361   treated (CS1000) exhibited a lower significant FBG than untreated type 2 diabetic rats at the end

362   of 3rd and 4th weeks ($P \leq 0.01$ and $P \leq 0.001$, respectively).

363   **Oral glucose tolerance test (OGTT)**

364   Figure 8 illustrates that blood glucose of untreated type 2 diabetic (D0) was significantly higher

365   than that of normal control (NC) at zero ($P \leq 0.01$), 30 ($P \leq 0.001$), 60 ($P \leq 0.001$), 90 ($P \leq 0.001$)

366   and 120 ($P \leq 0.001$). Conversely, blood glucose of glibenclamide-treated type 2 diabetic (GD) was

367   significantly lower than that of untreated type 2 diabetic at 30 ($P \leq 0.05$), 60 ($P \leq 0.001$), 90 ($P \leq$

368   0.001) and 120 ($P \leq 0.001$). However, blood glucose of low dose carob-treated (CS500) and high

369   dose carob-treated (CS1000) was significantly lower than that of untreated type 2 diabetic at 90 (P

370   ≤ 0.01 and $P \leq 0.001$, respectively) and 120 minutes ($P \leq 0.001$ and $P \leq 0.001$, respectively).

371   **Biochemistry and hemostatic assessment index of STZ-NAD diabetic animal model**

372   Table 6 showed that total protein of glibenclamide-treated type 2 diabetic (GD) and high dose

373   carob-treated (CS1000) was significantly higher than that of untreated type 2 diabetic (D0) ($P \leq$

374   0.01 and $P \leq 0.01$, respectively). On the other hand, serum amylase of untreated type 2 diabetic

375   was significantly lower than that of normal control (NC) ($P \leq 0.001$), glibenclamide-treated type

376   2 diabetic ($P \leq 0.001$), low dose carob-treated (CS500) ($P \leq 0.001$) and high dose carob-treated (P

377   ≤ 0.001). Serum FBG of untreated type 2 diabetic was higher than that of normal control ($P \leq$

378   0.001), while serum FBG of glibenclamide-treated type 2 diabetic and high dose carob-treated was

379   significantly lower than that of untreated type 2 diabetic ($P \leq 0.001$ and $P \leq 0.001$, respectively).

380   However, only insulin of untreated type 2 diabetic was less than that of normal control ($P \leq 0.001$).

381   In addition, HOMA-B of untreated type 2 diabetic was significantly less than that of normal control

382   ($P \leq 0.001$), glibenclamide-treated type 2 diabetic ($P \leq 0.001$), low dose carob-treated ($P \leq 0.05$)

383   and high dose carob-treated ($P \leq 0.01$), while HOMA-S and HOMA-IR showed a non-significant

384   difference.

385   **Histopathology of STZ-NAD diabetic animal model.**

386   Figure 9 showed that histological examination of pancreatic tissues exhibited that normoglycemic

387   untreated rats (A) showed a normal tissue morphological configuration and a larger islet (indicated

388   by red arrow) with high number of β-cells (indicated by yellow arrow), while the untreated type 2

389   diabetic (B) had the smallest islet and structural malformation with lowest number of β-cells. The

390   high dose carob-treated (E), low dose carob-treated (D) and glibenclamide-treated type 2 diabetic

391   (C) groups had comparatively larger islets with some abnormal structure and higher numbers of

392   β-cells were observed as compared to the untreated type 2 diabetic rats (B).

393   **Discussion**

394   The extraction solvents play a vital role in the pharmacological activities (Turkmen et al. 2006)

395   since the content of an extract is almost dependent on the extraction solvent, therefore, the current

396   study used methanol as a solvent of extraction for carob because it is one of the universal solvents

397   which is capable of extracting non- polar, semi-polar and polar constituents (AM Saghir et al.

398   2016).

399   Earlier reports indicated that carob pods contain considerable amounts of phenolic compounds

400   such as tannins (proanthocyanidins, gallotannins and ellagitannins), flavonoids, gallic acid, (+)-

401   catechin, (-)-epigallocatechin gallate, cinnamic acid, p-coumaric acid and quercetin glycosides

402   (Gulay et al. 2012; Marakis 1996; Owen et al. 2003; Rtibi et al. 2017a). Our findings revealed that

403   the methanolic extract of carob had a high content of total phenolic and flavonoids. In the current

404   study, the antioxidant activity of the methanolic carob extract using DPPH and FRAP assays was

405   evaluated prior to conduct its *in vivo* antioxidant activity to provide a preliminary evidence about

406   the antioxidant activity of the Yemeni cultivar of carob which has not been yet reported. Actually,

407   our findings of *in vitro* antioxidant activity indicated that the methanolic extract of carob produced

408   a scavenging activity against free radicals of DPPH which was higher than that was reported by

409   Rtibi et al. (2017b). Likewise, the methanolic extract of carob exhibited a ferric reducing

410   antioxidant power that accounted for 50% of those exhibited by quercetin and Trolox. The

411   antioxidant properties of the methanolic extract of carob maybe act through its ability to neutralize

412   reactive oxygen species and myeloperoxidase (Rtibi et al. 2015a; Rtibi et al. 2017b). Nonetheless,

413   the *in vitro* and *in vivo* antioxidant activity for most plants was not always the same (Kasote et al.

414   2015). Accordingly, further studies should consider the effect of methanolic extract of carob on

415   the *in vivo* antioxidant enzymatic system using pancreatic homogenate.

416   Universally, natural products are considered as one of the most common source of drugs (Newman

417   et al. 2003). Regardless of the effectiveness of natural products, those products should firstly be

418   assessed for their safety (Evans et al. 2001; Mounanga et al. 2015). In the present study, the safety

419   of the methanolic extract of carob was assessed *in vitro* on pancreatic β-cells and hepatocytes

420   followed by *in vivo* assessment of its acute toxicity on male and female rats for 14 days.

421   Accordingly, our findings indicated that the methanolic extract of carob was not toxic against

422   pancreatic β cells and hepatocytes as long as the viability of both cells remained more than 30%

423   after 24, 48 and 72 hours of exposure to carob (Jayash et al. 2017). Although *in vivo* acute oral

424   toxicity findings indicated a rise in ALP and urea of male rats as well as creatinine of female rats.

425   As it is known that liver is the main site of biotransformation of xenobiotics into metabolites which

426   could sometimes be hepatotoxic (Sturgill & Lambert 1997) inducing a rise in hepatic biomarkers

427   (ALP, AST and ALT) and histological abnormalities through destructing cell membrane and

428   structures of hepatocytes (Apaydin et al. 2017). However, ALP is ubiquitous in several body

429   organs and has several isoenzymes (Moss 1982). Accordingly, the increase in ALP of male rats

430   could be extrahepatic. Regarding the increase in creatinine of female rats, it is well-known that

431   creatinine is a measure of kidney functions, however, it was reported that creatinine is no longer

432   imprecise biomarker (Inker et al. 2012). Consequently, this study relied on the histological findings

433   in livers and kidneys sections of male and female rats to detect any direct toxicity evidence due to

434   the exposure to carob. Actually, the hsitological sections of liver of male and female rats showed

435   normal architecture of hepatocytes (at the level of their cell membrane, nuclei or even cytoplasm),

436   without necrotic lesions, inflammatory signs or fatty infiltrations. Similarly, histological sections

437   of kidneys of male and female rats showed normal architecture of tubules, Bowman's capsule,

438   Malpighian corpuscles.

439   Carob as a plant bears a great significance in the traditional medicine because of its various

440   pharmacological activities (Gulay et al. 2012; Rtibi et al. 2017a). Interesetingly, carob is a native

441   plant to Yemen and it is used as a traditional medicine to improve glycemic control in diabetics

442   long time ago (Battle & Tous 1997; Papakonstantinou et al. 2017). In point of fact, the previous

443   reports suggested that the physiological viscous property of soluble fiber of carob pods gum could

444   modify carbohydrates structure during digestion which can modify the rate of carbohydrate

445   degradation and control blood glucose levels (Barak & Mudgil 2014; Forestieri et al. 1989; Marles

446   & Farnsworth 1995). Consequently, this drew our attention to examine the *in vitro* inhibitory effect

447   of carob against α-amylase and α-glucosidase which are responsible for post-prandial conversion

448   of carbohydrates into glucose units (Etxeberria et al. 2012). Accordingly, to assess the glycemic

449   activity of the methanolic extract of carob, its *in vitro* glycemic activity was evaluated in term of

450   its inhibitory effect on α-amylase and α-glucosidase (Etxeberria et al. 2012). Our findings indicated

451   that carob could inhibit those enzymes, however, its IC_{50} against both enzymes was higher than

452   that of α-acarbose indicating that carob has *in vitro* hypoglycemic activity but it was not so

453   effective as α-acarbose. On the other hand, the *in vivo* glycemic activity of such extract was

454   assessed in T2D animal model. Actually, SD-rats were selected to create a type 2 diabetic animal

455   model (T2D) since those rats are most commonly used to investigate the glycemic effects of

456   medicinal plants (Furman 2015). In addition, SD-rats are more susceptible to become diabetic with

457   small dose of streptozotocin (STZ), and its ability to produce a dose-dependent destruction to

458   pancreatic β-cells (Abeeleh et al. 2009; Furman 2015). Nevertheless, several protocols have been

459   adopted to induce type 2 diabetes mellitus (T2DM). In this study, animals were injected with

460   intraperitoneal dose of 55 mg kg^{-1} of STZ followed by an intraperitoneal dose of 210 mg kg^{-1} of

461   nicotinamide, which prevented STZ from producing a complete destruction of pancreatic β-cells

462   in order to create T2D animal model of relative insulin secretion (Furman 2015). Our findings

463   indicated that STZ-NAD induced rats showed FBG of 12.74±0.99 mmol L^{-1} that fall within the

464   proposed range of FBG of T2DM (7.2-15.6 mmol L^{-1}) (Furman 2015; Zhang et al. 2009). Such

465   level of FBG was maintained through the time course of treatment indicating that the created T2D

466   animal model was stable.

467   Blood glucose of T2D rats after oral glucose challenge indicated that both low dose carob-treated

468   (CS500) and high dose carob-treated (CS1000) showed an antihyperglycemic effect after 90 and

469   120 minutes. However, only high dose carob-treated exerted a significant reduction in FBG at the

470   end of the 3rd and 4th week of treatment, which was emphasized by the significant increase of total

471   protein of high dose carob-treated due to alleviating the catabolic effect of the persistent

472   hyperglycemia on proteins (Ng et al. 2016), and the significant reduction in body weigh as a sign

473   of the resulted hypoglycemia (Pandit et al. 2010). The resulted hypoglycemic effect of high dose

474   carob-treated might be due to the low GIT value of carob and its high content of fibers which

475   provoked feeling of satiety (Papakonstantinou et al. 2017) or the polyphenols content of carob

476   could chelate sugars, lipids and fibers leading to reducing their intestinal absorption (Lattimer &

477   Haub 2010; Williamson 2013).

478   In normoglycemic control rats, the insulin level was significantly higher than that of untreated type

479   2 diabetic group (D0). Conversely, the insulin level of all treated and untreated diabetic rats was

480   non-significantly different, although it was suggested that carob could enhance insulin release

481   (Forestieri et al. 1989; Marles & Farnsworth 1995). For other *in vivo* mechanisms and pathways,

482   our study recommended further investigation on the effect of carob on GLP-1. On the other hand,

483   HOMA-B, as surrogate of pancreatic β-cells activity (Wallace et al. 2004) was significantly higher

484   than that of untreated type 2 diabetic due to that normoglycemic rats still had intact pancreatic β-

485   cells since they were not exposed to the destructive effect of streptozotocin (Furman 2015), which

486   was emphasized with their normal findings of the pancreatic histological sections. Similarly,

487   HOMA-S and HOMA-IR of all treated and untreated diabetic rats as well as normal control

488   showed a non-significant difference. Actually, the carob pod is rich in D-pinitol compound (Tetik

489   et al. 2011) which could play an insulin-like role in improving the insulin sensitivity (Gao et al.

490   2015). Nonetheless, the used model was streptozotocin-nicotinamide diabetes induced (insulin

491   deficient model) rather than insulin resistant induced model (Furman 2015). On the other hand,

492   the slight increase of insulin in of glibenclamide-treated type 2 diabetic (GD) group was due to

493   that glibenclamide is a well-known insulin tropic agent (Chan & Colagiuri 2015), while the slight

494   increase in insulin of high dose carob-treated (CS1000) could be independent of insulin

495   (Papakonstantinou et al. 2017). Perhaps carob provided a protection to the remaining β-cell from

496   destruction owing to its antioxidant activity (Coskun et al. 2005; Kaneto et al. 1999; Nasri et al.

497   2015). particularly that our findings of the scavenging activity of carob against free radicals of

498   DPPH was indicated. As mentioned above, however, the findings of the *in vitro* antioxidant

499   activity of carob extract could not be extrapolated to reflect the *in vivo* antioxidant activity (Kasote

500   et al. 2015). Nonetheless, low dose carob-treated and high dose carob-treated showed a higher

501   significant value of HOMA-B than that of untreated type 2 diabetic (D0) indicating that low dose

502   carob-treated (CS500) and high dose carob-treated (CS1000) still had active insulin secreting β-

503   cells, which could be supported by higher number of β-cells that was observed in the histological

504   pancreatic sections of low dose carob-treated (D) and high dose carob-treated (E) as compared to

505   untreated type 2 diabetic (B).

506 **CONCLUSION**

507 Carob pod did not cause acute systemic toxicity and showed *in vitro* antioxidant effects. On the

508 other hand, the *in vitro* ex-pancreatic hypoglycemic effect of the methanolic extract of carob pods

509 was evident through inhibiting α-amylase and α-glucosidase. Interestingly, high dose of carob

510 exhibits an *in vivo* antihyperglycemic activity and warrants further in depth study to identify the

511 bioactive constituents in the potential methanolic extract of carob pods.

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688

Total phenolic and flavonoid with the antioxidant activities of the methanolic extract of carob.

The table demonstrated that the phenolic content (y = 0.0075x - 0.0123, R^2 = 0.9817) was 127.02 ± 7.18 mg GAE g^{-1}, while the flavonoid content (y = 0.0023x - 0.0165, R^2 = 0.9979) was 49.74 ± 0.88 mg QE g^{-1}, which means that flavonoid content approximately accounted for 39.2% of the total phenolic content.

1   **Table 1** Total phenolic and flavonoid with the antioxidant activities of the methanolic extract of
2   Carob

	Total phenolic content (mg GAE g^{-1})	Total flavonoid Content (mg QE g^{-1})	DPPH radical IC$_{50:}$ μg mL^{-1}	FRAP assay μmoL FeSo$_4$ mg^{-1}
Carob extract	127.02 ± 7.18	49.74 ± 0.88	11.23 ±0.47	3191.67 ± 54.34
Quercetin	-	-	6.64 ± 0.07	6565.17 ± 678.75
Trolox	-	-	9.46 ± 0.14	6433 ± 216.49

3   Values were expressed as mean±SE, (n=3).

Correlation coefficients of phenolic and flavonoid contents against antioxidant assays.

The Pearson correlations analysis displayed that the total phenolic content of carob methanolic extract had a strong positive correlation with FARP assay (r= 0.918, *P*<0.01) and a strong negative with DPPH radical (r= -0.910, *P*<0.01). Likewise, total flavonoid content of carob methanolic extract reflects a strong positive correlation with FRAP asaay (r= 0.886, *P*<0.01) and a strong negative correlation with DPPH radical (r= -0.867, *P*<0.01).

1   **Table 2** Correlation coefficients of phenolic and flavonoid contents against antioxidant assays

Methanolic carob extract	FRAP assay	DPPH radical
Total phenolic content	0.918**	-0.910**
Total flavonoid content	0.886**	-0.867**

3   ** means correlation is significant at the 0.01 levels (2-tailed).

Body weight as well as absolute and relative organ weight of female and male rats.

The table showed that no significant difference was observed in body weight of male and female rats. Like body weight, absolute liver, kidney, heart and pancreas weights, as well as relative liver, kidney, heart and pancreas weights of male and female rats were non-significantly different.

Table 3 Body weight as well as absolute and relative organ weight of female and male rats

	Females			Males		
	NC	**CS2000**	**CS5000**	**NC**	**CS2000**	**CS5000**
BW_0	230.00±15.16	226.67±18.61	230.83±13.93	356.67±22.73	363.33±39.33	360.83±36.66
BW_1	246.33±16.08	251.67±24.83	260.83±14.63	410.00±24.70	407.50±41.32	402.50±37.52
BW_2	250.00±20.73	244.17±21.77	237.50±15.73	399.17±25.3	406.67±39.96	391.67±40.21
ALW	7.88±0.75	7.53±0.86	7.40±1.12	12.70±0.83	12.23±1.72	12.2±1.1
AKW	1.78±0.25	1.55±0.19	1.65±0.19	2.77±0.23	2.65±0.31	2.70±0.36
AHW	0.85±0.15	0.85±0.10	0.80±0.09	1.13±0.16	1.35±0.19	1.22±0.15
APW	1.22±0.15	1.15±0.19	1.05±0.24	1.47±0.29	2.00±0.59	1.78±0.50
RLW	0.032±0.001	0.031±0.002	0.031±0.003	0.032±0.002	0.030±0.002	0.031±0.001
RKW	0.007±0.001	0.006±0.0003	0.007±0.001	0.007±0.001	0.007±0.0003	0.007±0.0004
RHW	0.003±0.0005	0.004±0.0003	0.003±0.0003	0.003±0.0004	0.003±0.0003	0.003±0.0002
RPW	0.005±0.0003	0.005±0.0005	0.004±0.0007	0.004±0.001	0.005±0.001	0.004±0.001

NC: normal control, CS2000: 2000 mg kg^{-1} carob-treated, CS5000: 5000 mg kg^{-1} carob-treated. BW0, 1 and 2: body weight before treatment as well as at the end of 1st and 2nd week of treatment, respectively. ALW: absolute liver weight, AKW: absolute kidney weight, AHW: absolute heart weight, APW: absolute pancreas weight, RLW: relative liver weight, RKW: relative kidney weight, RHW: Relative heart weight, RPW: Relative pancreas weight. Values are expressed as mean±SD, (n=6).

Biochemical parameters for male and female rats.

The table showed that only alkaline phosphatase and urea of CS5000 were significantly increased as compared to normal control (NC). In female rats, only serum creatinine showed significant increased as compared to noramal control.

1   **Table 4** Biochemical parameters for male and female rats.

	Females			Males		
	NC	**CS2000**	**CS5000**	**NC**	**CS2000**	**CS5000**
ALP	73.60±3.21	76.20±20.99	93.60±16.13	76.80±11.97	79.00±12.25	142.00± 7.87**
AST	93.80±10.03	126.80±32.58	132.60±38.49	101.60±13.46	121.20±14.57	100.60±25.77
ALT	59.80±4.82	53.20±11.90	57.80±1.64	34.00±4.74	34.80±3.77	40.20±5.93
TP	30.00±1.87	31.60±7.02	35.20±2.86	7.26±0.42	7.28±0.38	7.06±0.46
Cr	6.12±0.52	7.36±1.68	9.62±1.93**	51.80±6.98	53.20±2.68	52.00±2.55
Ur	7.58±0.26	7.58±0.57	7.00±0.53	7.06±1.21	7.36±1.07	11.20±1.18*

3   NC: normal control, CS2000: 2000 mg kg^{-1} carob-treated, CS5000: 5000 mg kg^{-1} carob-treated. ALP:
4   alkaline phosphatase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, TB: total bilirubin,
5   Cr: creatinine, Ur: urea. Superscript *, **: symbolized a significant difference at either $P < 0.05$ or $P \leq 0.01$
6   versus normal control. Values were expressed as mean ± SD, (n=5).

Body weight as well as absolute and relative pancreas weight of STZ-NAD diabetic animal model.

The table demonstrated that body weight of CS500 and CS1000 was significantly reduced more than untreated type 2 diabetic rats (D0). On the other hand, absolute pancreas weight (APW) and relative pancreas weight (RPW) of CS1000 were significantly lower than that of untreated type 2 diabetic rats group.

1 **Table 5** Body weight as well as absolute and relative pancreas weight of STZ-NAD diabetic
2 animal model.

	NC	D0	GD	CS500	CS1000
BW0	287.2±32.31	289.2±17.42	282.3±22.84	273.3±16.33	288.7±20.71
BW4	360.3±30.92	333.7±14.53	306.0±8.31	296.8±13.82*	281.3±25.02**
APW	2.34±0.16	2.02±0.33	1.79±0.14	1.62±0.36	1.27±0.37***
RPW	0.0066±0.001	0.0063±0.001	0.0059±0.001	0.0055±0.001	0.0044±0.001**

3 NC: normoglycemic untreated rats, D0: untreated type 2 diabetic rats, GD: glibenclamide-treated type 2 diabetic,
4 CS500: 500 mg kg^{-1} of carob-treated type 2 diabetic, CS1000:1000 mg kg^{-1} of carob-treated type 2 diabetic, BW0
5 and 4: initial body and final body weight, respectively. APW: absolute pancreas weight, RPW: relative pancreas
6 weight. Superscript **, ***: denoted a significant difference at $P \leq 0.01$ and $P \leq 0.001$ versus D0. Values were
7 expressed as mean±SE, (n=6).

Biochemistry and hemostatic assessment index of STZ-NAD diabetic animal model

The table showed that the total protein of glibenclamide-treated type 2 diabetic (GD) and CS1000 was significantly higher than that of untreated type 2 diabetic (D0) . On the other hand, serum amylase of untreated type 2 diabetic was significantly lower than that of normal control, glibenclamide-treated type 2 diabetic, CS500 and CS1000. Serum FBG of untreated type 2 diabetic was higher than that of normal control (NC), while serum FBG of glibenclamide-treated type 2 diabetic and CS1000 was significantly lower than that of untreated type 2 diabetic. However, only insulin of untreated type 2 diabetic was less than that of normal control . In addition, HOMA-B of untreated type 2 diabetic was significantly less than that of normal control, glibenclamide-treated type 2 diabetic, CS500 and CS1000, while HOMA-S and HOMA-IR showed a non-significant difference.

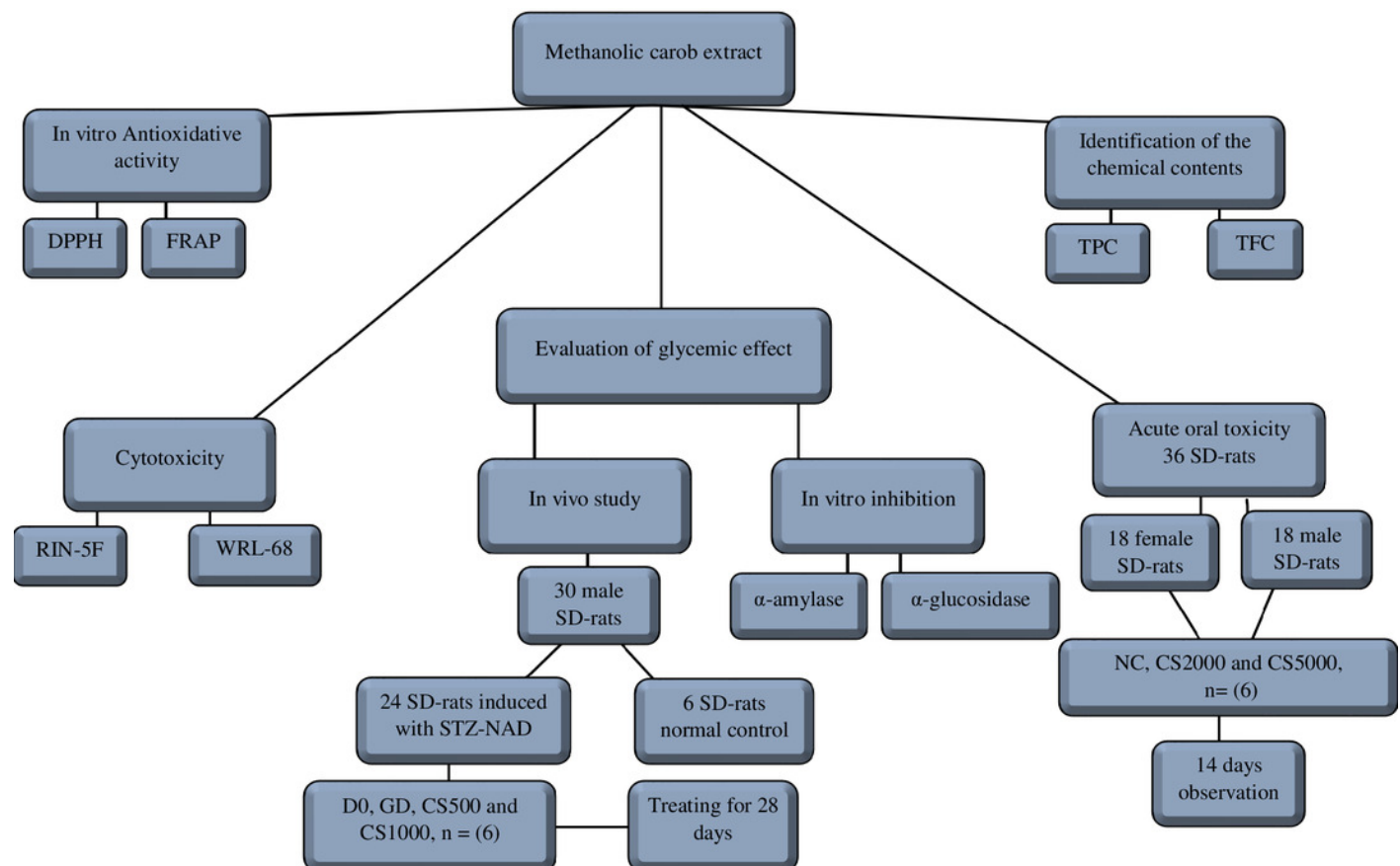
	NC	D0	GD	CS500	CS1000
TP g L^{-1}	90.60±6.40	77.10±12.71	100.52±7.06**	86.68±10.37	101.16±8.65**
Amy U L^{-1}	2891.80±22.1	1717.00±24.81$^{\#\#\#}$	2757.60±18.91***	2863.00±21.20***	2910.60±22.42***
TG mmol L^{-1}	0.52±0.24	0.63±0.41	0.81±0.27	0.77±0.24	0.84±0.32
TC mmol L^{-1}	2.70±0.24	2.52±1.07	3.16±0.30	2.82±0.49	3.36±0.69
FBG mmol L^{-1}	4.90±0.41	13.51±1.23$^{\#\#\#}$	6.50±0.40***	12.34±0.90	10.35±0.85***
IN mU L^{-1}	15.55±1.26	10.60±0.95$^{\#\#\#}$	13.15±2.22	11.26±1.25	12.44±1.87
HOMA-β	163.28±24.46	17.43±0.93$^{\#\#\#}$	82.48±12.41***	22.85±2.95*	33.88±7.39**
HOMA-S	51.12±4.32	55.73±5.89	56.80±8.33	56.43±7.42	54.03±7.49
HOMA-IR	1.97±0.19	1.78±0.22	1.80±0.31	1.80±0.23	1.88±0.26

1 **Table 6** Biochemistry and hemostatic assessment index of STZ-NAD diabetic animal model.

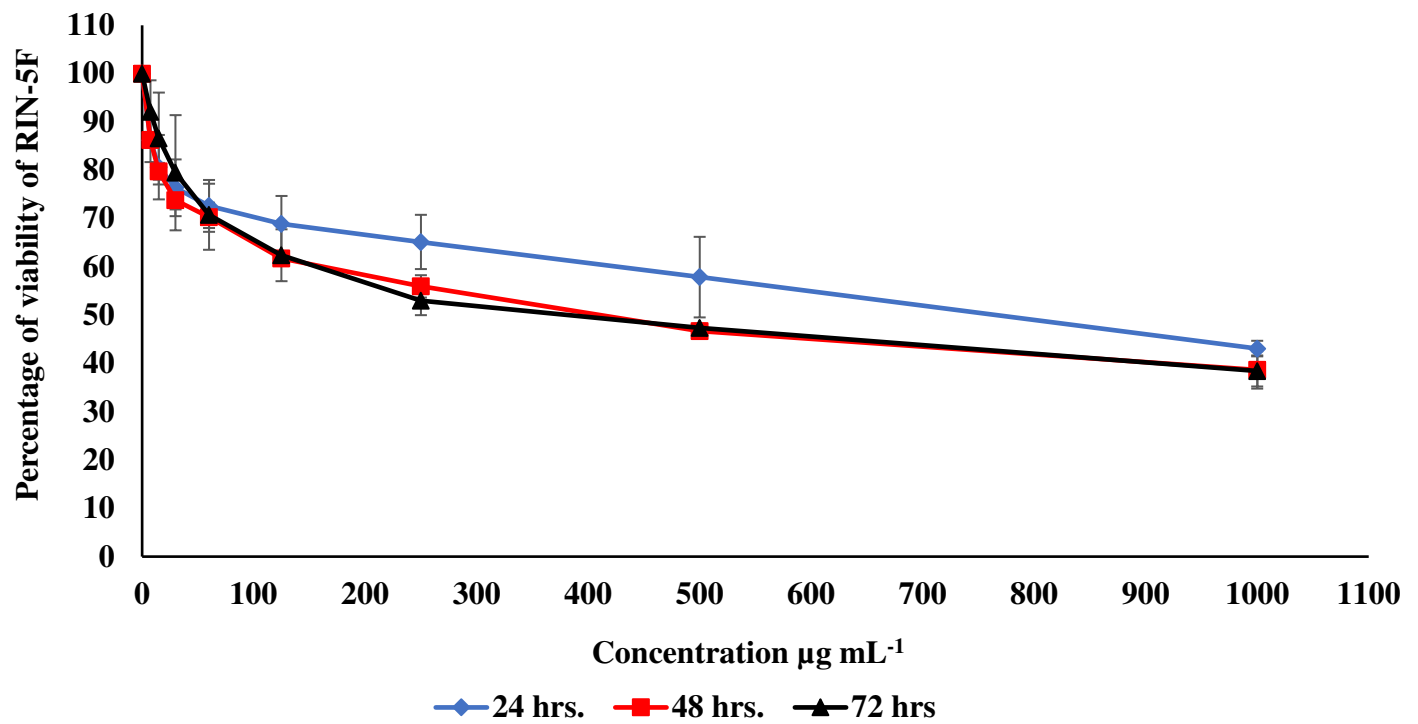
2 NC: normoglycemic untreated rats, D0: untreated type 2 diabetic, GD: glibenclamide-treated type 2 diabetic, CS500: 500 mg kg^{-1} of carob-treated type 2 diabetic,
3 CS1000:1000 mg kg^{-1} of carob-treated type 2 diabetic, TP: total protein, Amy: amylase, FBG: fasting blood glucose, IN: insulin, HOMA-β: βeta cell function,
4 HOMA-S: insulin sensitivity, HOMA-IR: insulin resistance. Superscript $^{\#\#\#:}$ denoted a significant difference at $P \leq 0.001$ versus normal control. Superscript $^{*, **,}$
5 $^{***:}$ denoted a significant difference at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ versus D0. Values were expressed as mean±SD, (n=6).

Figure 1

In vivo and *in vitro* experimental model design of study

The figure demonstrated the flow chart of the experimental design.

Effect of the methanolic extract of carob on viability of pancreatic RIN-5F cells.

The figure illustrated that the percentage of viability of RIN-5F cells after 24, 48 and 72 hours of exposure to the methanolic extract of carob. The percentage of viability was upper than 30%.

Values were expressed as mean±SD, (n=3).

Effect of the methanolic extract of carob on viability of WRL 68 cells.

The figure demonstrated the percentage of viability of WRL 68 cells after 24, 48 and 72 hours of exposure to the methanolic extract of carob. The percentage of viability was upper than 30%.Values were expressed as mean±SD, (n=3).

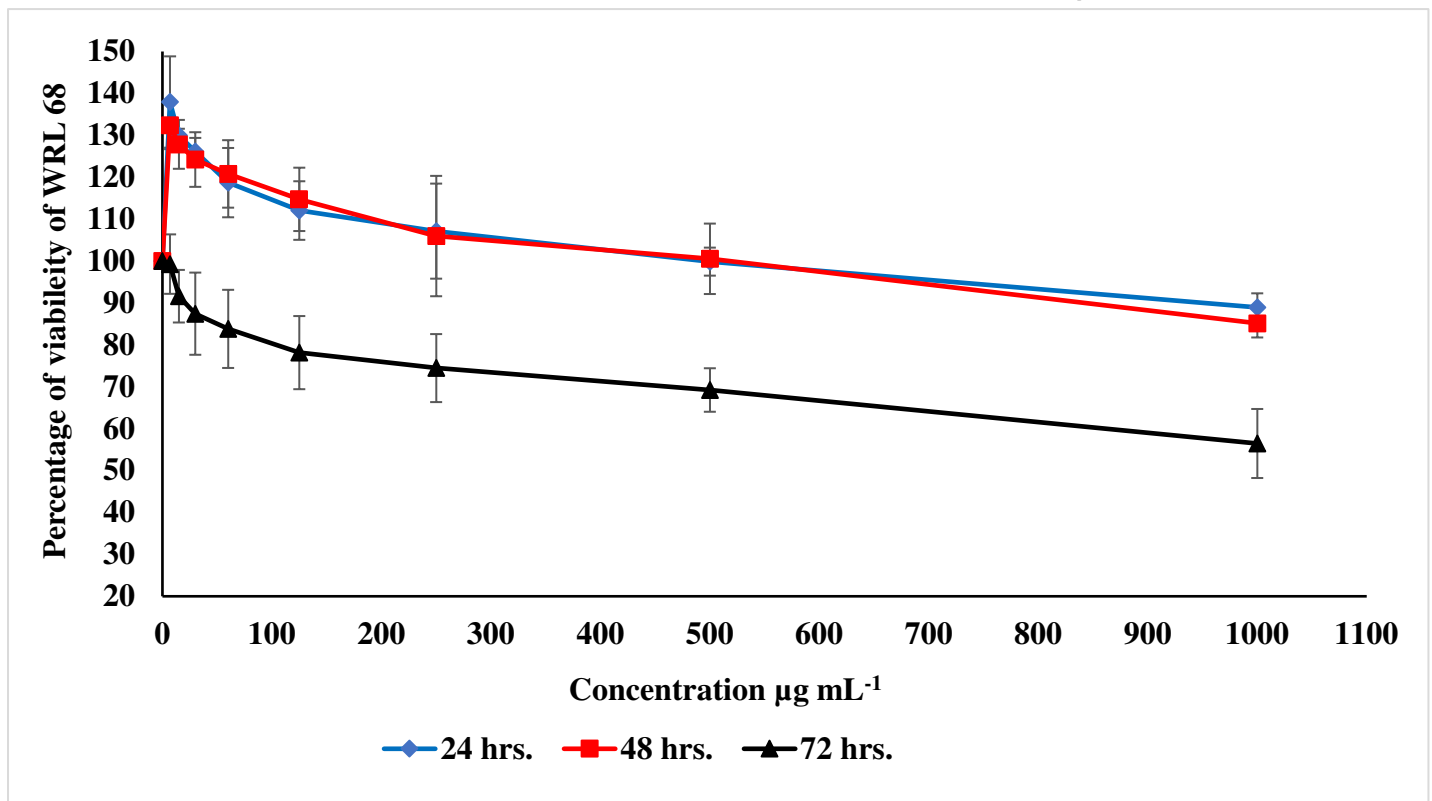
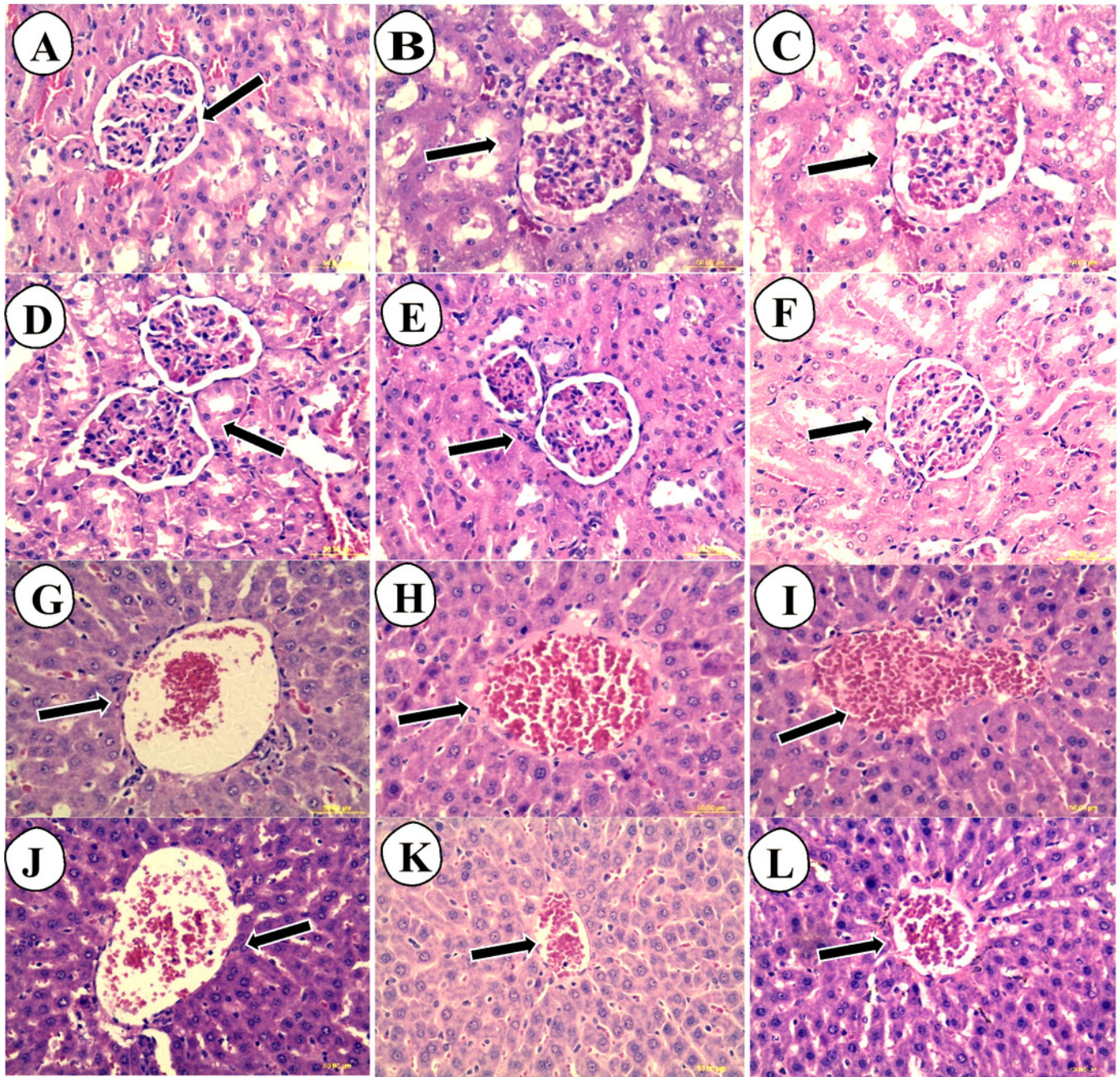
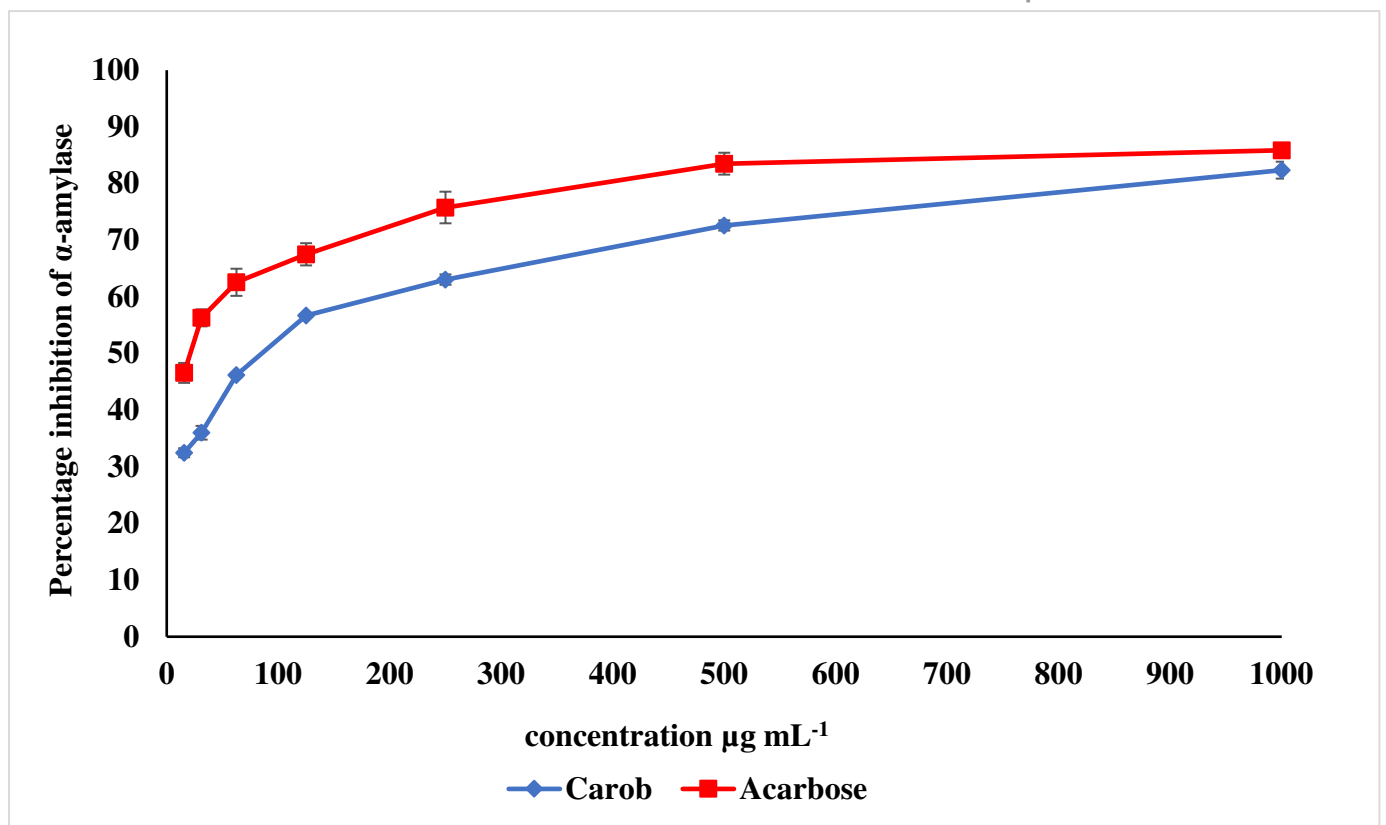



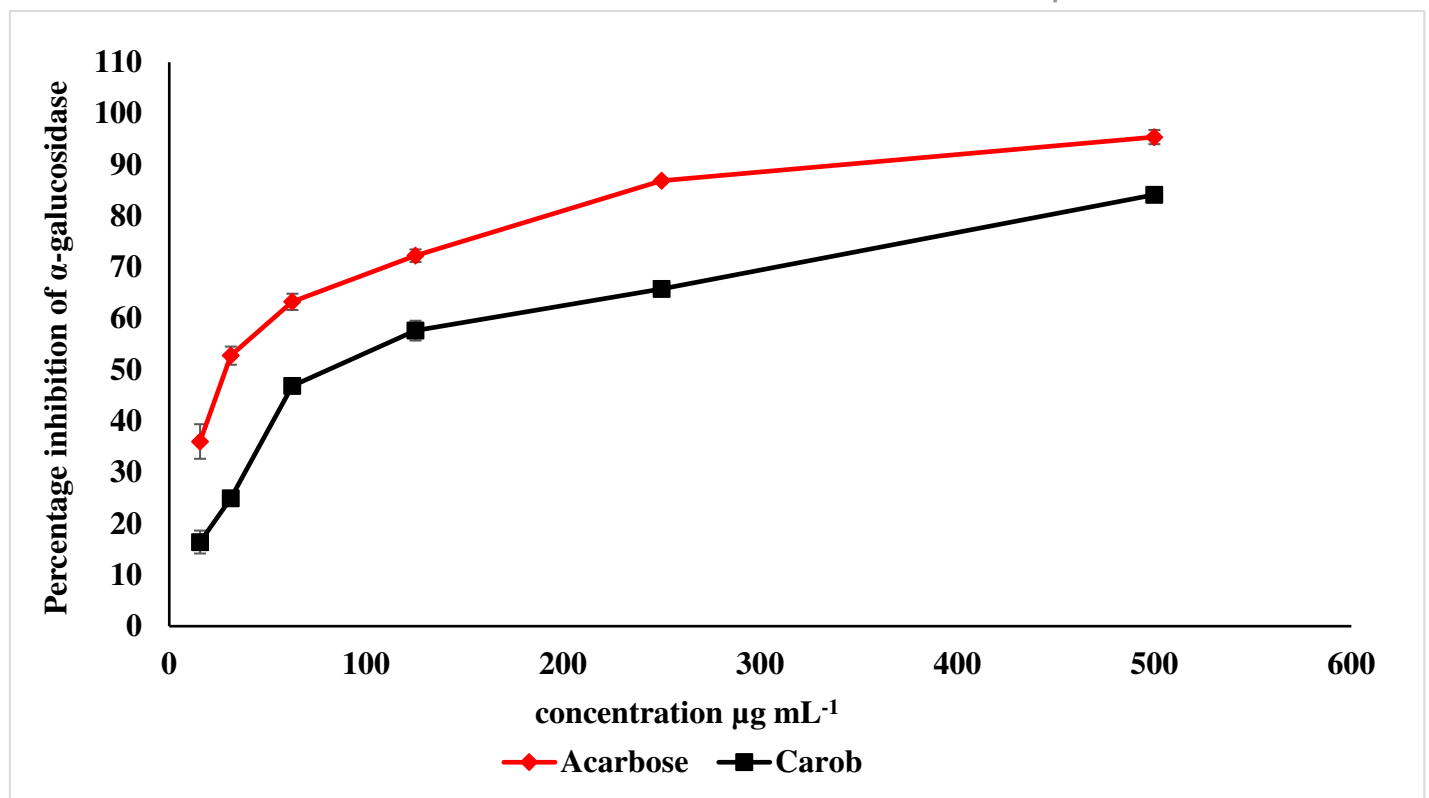
Figure 4

Hematoxylin and eosin stained histopathological sections of livers and kidneys of male and female SD-rats.

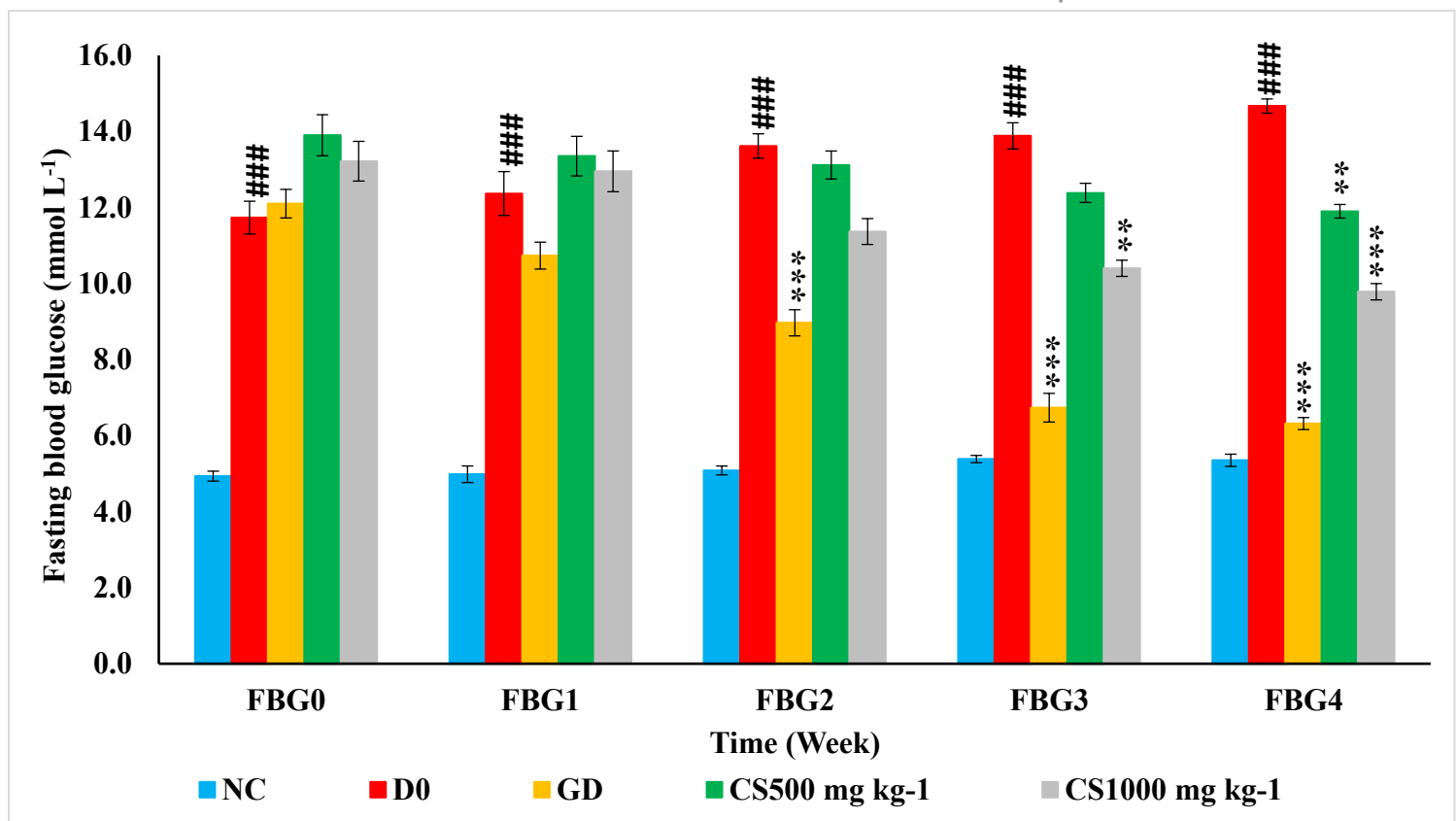
The figure illustrated the hematoxylin and eosin stained histopathological sections of livers and kidneys of male and female SD-rats.

A, B and C: normal control (NC), 2000 mg kg^{-1} carob-treated (CS2000) and 5000 mg kg^{-1} carob-treated (CS5000) of male kidney sections, respectively. D, E and F: normal control (NC), 2000 mg kg^{-1} carob-treated (CS2000) and 5000 mg kg^{-1} carob-treated (CS5000) of female kidney sections, respectively. G, H and I: normal control (NC), 2000 mg kg^{-1} carob-treated (CS2000) and 5000 mg kg^{-1} carob-treated (CS5000) of male liver sections, respectively. J, K and L: normal control (NC), 2000 mg kg^{-1} carob-treated (CS2000) and 5000 mg kg^{-1} carob-treated (CS5000) of female liver sections, respectively. Liver sections of G, H, I, J, K and L, showed similar normal architecture of hepatocytes at the level of their cell membrane, nuclei or even cytoplasm without any abnormalities such as congestion of portal veins, necrotic lesions, inflammatory signs or fatty infiltrations. Similarly, kidney sections of all groups similar normal architecture of tubules, Bowman's capsule, Malpighian corpuscles without any abnormal findings (magnification X 40).

Inhibitory effect of the methanolic extract of carob against α-amylase.

The figure demonstrated the percentage of inhibition of the methanolic carob extract and α-acarbose against against α-amylase. The IC_{50} of carob methanolic extract (92.99 ± 0.22 μg mL^{-1}) was higher than that of α-acarbose (23.33 ± 0.73 μg mL^{-1}) against α-amylase.

Inhibitory effect of the methanolic extract of carob against α-glucosidase.

The figure showed the percentage of inhibition of the methanolic carob extract and α-acarbose against α-glucosidase. The IC_{50} of the methanolic carob extract (97.13±4.11 µg mL^{-1}) against α-glucosidase was higher than that of α-acarbose (27.05±0.99 µg mL^{-1}).

Weekly fasting blood glucose of STZ-NAD diabetic animal model .

The figure showed that the fasting blood glucose (FBG) of normal control group (NC) was higher significant than untreated type 2 diabetic rats (D0) at the baseline as well as the end of the 1st, 2nd, 3rd and 4th week of treatment. In addition, FBG of glibenclamide-treated type 2 diabetic (GD) was significantly lower than D0 at the end of the 2nd, 3rd, and 4th weeks. Moreover, CS500 showed a lower significant FBG than D0 only at the end of 4th week while CS1000 exhibited a lower significant FBG than D0 at the end of 3rd and 4th weeks.

NC: normoglycemic untreated rats, D0: untreated type 2 diabetic, GD: glibenclamide-treated type 2 diabetic, CS500: 500 mg kg^{-1} of carob-treated type 2 diabetic, CS1000: 1000 mg kg^{-1} of carob-treated type 2 diabetic. FBG$_0$, 1, 2, 3 and 4: fasting blood glucose at baseline as well as at the end of the 1st, 2nd, 3rd and 4th week of treatment, respectively. Superscript ###: denoted a significant difference at $P \leq 0.001$ versus normal control. Superscript **, ***: denoted a significant difference at $P \leq 0.01$ and $P \leq 0.001$ versus D0. Values were expressed as mean±SE, (n=6).

Oral glucose tolerance test of STZ-NAD diabetic animal model

The figure illustrated that the blood glucose of untreated type 2 diabetic rats (D0) was significantly higher than that of normal control (NC) at zero, 30, 60, 90 and 120. Conversely, blood glucose of glibenclamide-treated type 2 diabetic (GD) was significantly lower than that of untreated type 2 diabetic at 30, 60, 90 and 120. However, blood glucose of CS500 and CS1000 was significantly lower than that of untreated type 2 diabetic at 90 and 120 minutes.

NC: normoglycemic untreated rats, D0: untreated type 2 diabetic, GD: glibenclamide-treated type 2 diabetic, CS500: mg kg^{-1} of carob-treated type 2 diabetic, CS1000:1000 mg kg^{-1} of carob-treated type 2 diabetic. Superscript $^{\#\#, \#\#\#:}$ denoted a significant difference at $P \leq 0.01$ and $P \leq 0.001$ versus normal control. Superscript $^{*, **, ***:}$ denoted a significant difference at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ versus D0. Values were expressed as mean±SE, (n=6).

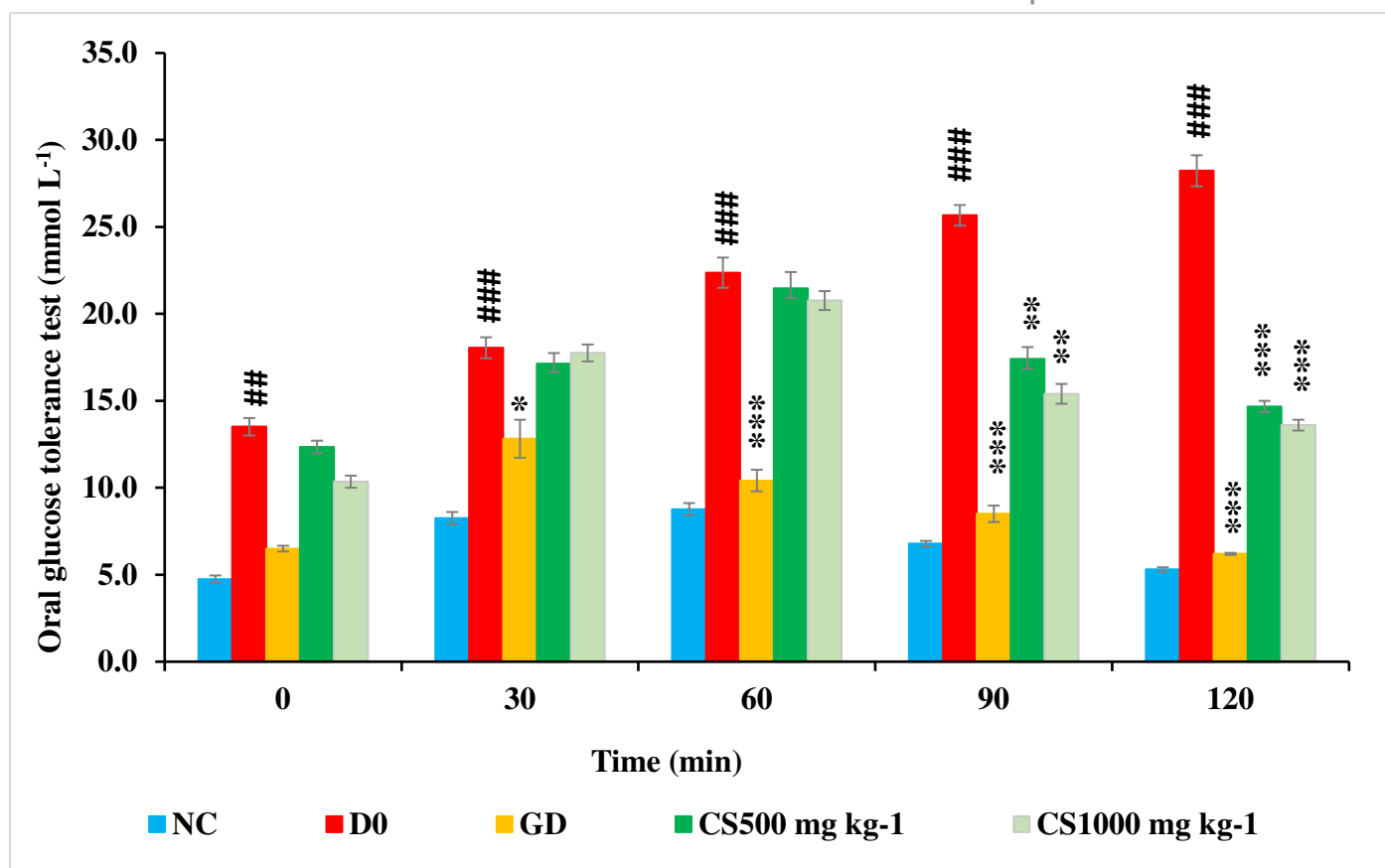



Figure 9

Hematoxylin and eosin stained nicotinamide streptozotocin-induced diabetic rats.

The figure showed that the histological examination of pancreatic tissues exhibited that normoglycemic untreated rats (A) showed a normal tissue morphological configuration and a larger islet (indicated by red arrow) with high number of β-cells (indicated by yellow arrow), while the untreated type 2 diabetic (B) had the smallest islet and structural malformation with lowest number of β-cells. In addition, high dose carob-treated type 2 diabetic CS1000 (E), low dose carob-treated type 2 diabetic CS500 (D) and glibenclamide-treated type 2 diabetic (C) had comparatively larger islets with some abnormal structure and higher numbers of β-cells were observed as compared to the untreated type 2 diabetic (B).

A: normoglycemic untreated rats (NC), B: untreated type 2 diabetic (D0), C: glibenclamide-treated type 2 diabetic (GD), D: 500 mg kg^{-1} of carob-treated type 2 diabetic (CS500), E:1000 mg kg^{-1} of carob-treated type 2 diabetic (CS1000). Red and yellow arrows denoted to pancreatic islets and β-cells, respectively, (magnification × 40).