Synergistic effect of quercetin and quinic acid by alleviating structural degeneration in the liver, kidney and pancreas tissues of STZ-induced diabetic rats: A mechanistic study

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Abstract
The aim of this study was to investigate the synergistic effects of quercetin (QE) and quinic acid (QA) on a STZ-induced diabetic rat model to determine their potential role in alleviating diabetes and its associated complications. In our study design, diabetic rats were treated with single and combined doses of QE and QA for 45 days to analyse their effects on liver, kidney and pancreas tissues. The study result showed that QE and QA treated groups down-regulated hyperglycaemia and oxidative stress by up-regulating insulin and C-peptide levels. Moreover, histological observations of the liver, kidney and pancreas of diabetic rats treated with single and combined doses of QE and QA showed a significant improvement in the structural degeneration. Interestingly, the combination dose of QE and QA (50 mg/kg) exhibited maximum inhibition of the pro-apoptotic protein Bax expression and demonstrate enhancement of the anti-apoptotic protein Bcl-2 expression in the kidney tissues, suggesting a protective role in the kidneys of diabetic rats. Taken together, these results indicates the synergistic effects of QE and QA in ameliorating hyperglycaemia, hyperlipidemia and insulin resistance in diabetic rats and therefore, open a new window of research on the combinatorial therapy of flavonoids.

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1. Introduction
The most common metabolic disorder caused by total or relative insulin insufficiency is diabetes mellitus (DM), which is characterised by hyperglycaemia and impaired metabolism of carbohydrates, fats, and proteins (Pooja and Samanta, 2011). DM is characterised by high blood glucose levels, which result from insulin resistance in peripheral tissues or impaired insulin synthesis in the pancreas (De Silva et al., 2012). High blood glucose is also associated with the generation of reactive oxygen species and consequent oxidative damage in the liver, kidneys, and pancreas (Mohamed et al., 1999). According to the World Health Organisation (WHO), DM is a disease with continuous increasing prevalence (WHO, 2006). This disease affects people worldwide, but the percentages of diabetics in the global population are particularly high in Asia and Europe (World Diabetes Foundation (WDF, 2010). Thus, the practice of herbal medicine for the treatment of DM is nearly universal among non-industrialised societies (Singh et al., 2001). According to an estimation published by the WHO in 2008, approximately 80% of diabetic patients in the world's population presently rely on herbal medicine for their successive treatments. Flavonoids are ubiquitously present in common plant-based food items and beverages. The first therapeutically beneficial role of dietary flavonoids was demonstrated by Rusznyak and Szent-Györgyi (1936). Medicinal plants may serve as a good source for the design of drugs as anti-diabetic agents, and much attention has been paid to formulations of herbal medicine (Vishwakarma et al., 2010). Potent antioxidant properties may be exhibited by many flavonoids (Rice-Evans, 2001; Taha et al., 2014). In addition, the implication of oxidative stress in the pathogenesis of DM has been proposed to not only generate oxygen free radicals but also alter antioxidant enzymes and the formation of lipid peroxides (Ceriello and Motz, 2004). Thus, defence mechanisms against free...
radicals, such as superoxide dismutase (SOD) and catalase (CAT), the activities of which contribute to the elimination of superoxide, hydrogen peroxide, and hydroxyl radicals, have been investigated. In addition, because DM is considered to be a free radical-mediated disease, there has been renewed interest in the use of flavonoids, including the flavonol quercetin, in diabetes research (Sanders et al., 2001; Vessel et al., 2003).

Quercetin is a naturally occurring flavonoid that is found in onions and other vegetables and may demonstrate beneficial effects on disease prevention. It has been reported that quercetin exerts protective effects against β-cell damage in diabetes and is capable of decreasing serum glucose levels in diabetic rats (Coskun et al., 2005). It has also been reported that quercetin can protect many types of tissue or organs, including the liver (Yokoyama et al., 2009), kidneys (Yousef et al., 2010) and pancreatic gland (Coskun et al., 2005), against oxidative damage. Quinic acid derivatives, which are polyphenol-rich compounds, consist of a large family of esters formed between quinic acid and one or more of several phenylpropanoic acids, such as caffeic acid, ferulic acid, coumaric acid, sinapic acid, and cinnamic acid (Lee et al., 2013). Quinic acid derivatives exhibit various beneficial effects, including antioxidant, anti-inflammatory, anti-HIV, anti-hepatitis B virus, hypoglycaemic, and hepatoprotective activities, as well as the inhibition of mutagenesis and carcinogenesis (Farah and Donangelo, 2006; Gorzalczany et al., 2008).

However, there has been no further assessment of the anti-hyperglycaemic effect of the combined administration of quercetin and quinic acid on diabetic rats. Thus, this study aimed to investigate the potential mechanisms underlying the anti-hyperglycaemic effect of these two compounds in an STZ-induced diabetic animal model.

2. Materials and methods

2.1. Chemicals

Streptozotocin (STZ) was obtained from Sigma Aldrich (USA). Glibenclamide was acquired from the University Malaya Medical Centre (UMMC). Quinic acid and quercetin were purchased from Sigma-Aldrich USA.

2.2. Animal stock

Healthy, mature Sprague Dawley (SD) male rats were provided by the Experimental Animal House of the Faculty of Medicine of University of Malaya (FM/27/7/2014/MMBA (8)). Rats weighing between 200 and 225 g were maintained in wire-bottomed cages at 25 ± 2 °C, administered tap water and a standard pellet diet, and exposed to a 12-h light/12-h dark cycle at 50–60% humidity in an animal room. Throughout the experiments, all of the animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals,” which was prepared by the National Academy of Sciences and published by the National Institute of Health.

2.3. Diabetes mellitus induction by STZ

Diabetes was induced in rats by the intraperitoneal (i.p.) injection of freshly prepared STZ at a dose of 45 mg/kg of body weight. Three days after the STZ injection, the glucose level of blood collected from the tail vein was determined, and hyperglycaemic rats (blood glucose level >16.7 mmol/L) were considered diabetic animals, which was prepared by the National Academy of Sciences and published by the Organisation for Economic Co-operation and Development (OECD, 1998). Male and female healthy adult Sprague Dawley rats were used in this study. The rats were fasted overnight, divided into three groups (n = 6), and orally fed a combination of quercetin and quinic acid at doses of 10, 100, and 500 mg/kg of body weight (bw). Quercetin and quinic acid were dissolved in distilled water and fed to the animals; the control groups were administered distilled water only. The rats were continuously observed during the first hour, every 1 h for the next 4 h, and every 24 h for up to 14 days for any physical signs of toxicity, such as writhing, gasping, palpituation, decreased respiratory rate, and lethality.

2.4. Experimental design

The animals were randomly divided into nine groups containing six rats per group as follows:

Group 1: Normal control rats fed normal saline NaCl for 45 days: NC.
Group 2: Diabetic control rats treated with saline solution for 45 days: DC.
Group 3: Diabetic positive control rats treated with glibenclamide for 45 days: PC.
Group 4: Diabetic rats treated with quercetin (50 mg/kg/d) for 45 days: QEAa.
Group 5: Diabetic rats treated with quercetin (100 mg/kg/d) for 45 days: QEb.
Group 6: Diabetic rats treated with quinic acid (50 mg/kg/d) for 45 days: QAa.
Group 7: Diabetic rats treated with quinic acid (100 mg/kg/d) for 45 days: QAa.
Group 8: Diabetic rats treated with quercetin (50 mg/kg) + quinic acid (50 mg/kg) for 45 days: QEA + QAa.
Group 9: Diabetic rats treated with quercetin (100 mg/kg) + quinic acid (100 mg/kg) for 45 days: QEB + QAa.

2.5. In vivo acute toxicity test

The acute toxicity test was performed according to the guidelines delineated by the Organisation for Economic Co-operation and Development (OECD, 1998). Male and female healthy adult Sprague Dawley rats were used in this study. The rats were fasted overnight, divided into three groups (n = 6), and orally fed a combination of quercetin and quinic acid at doses of 10, 100, and 500 mg/kg of body weight (bw). Quercetin and quinic acid were dissolved in distilled water and fed to the animals; the control groups were administered distilled water only. The rats were continuously observed during the first hour, every 1 h for the next 4 h, and every 24 h for up to 14 days for any physical signs of toxicity, such as writhing, gasping, palpituation, decreased respiratory rate, and lethality.

2.6. Measurement of blood glucose

The weekly blood glucose levels of samples obtained from the tail vein of rats in all of the groups were measured using reagent strips (Accu-Check Active Glucose test strips, Roche, Germany) with a glucometer (Accu-Check Active, Roche, Germany).

2.7. Biochemical parameters assessment

After 45 days of study period all the animals were sacrificed and blood was collected for the estimation of insulin, C-peptide, Glycated haemoglobin, Albumin, lipid profile, total antioxidant status (MDA, SOD and CAT). After blood collection, it was allowed to clot and centrifuged at 4000 g for 10 min and the serum obtained was used for the further measurement of all the markers.

2.8. Lipid peroxidation measurement

Serum lipid peroxidation was evaluated by measuring TBARS according to a modified method of Levine et al. (1990). Briefly, 0.2 mL of serum samples were added to the reaction mixture containing 1 mL of 1% orthophosphoric acid and 0.25 mL alkaline solution of thiobarbituric acid (final volume 2 mL), followed by 45 min heating at 95 °C. After cooling, samples and standards of malondialdehyde were read at 532 nm against the blank of the standard curve. The results were expressed as nmol MDA/μg protein.

2.9. Antioxidant enzymes (SOD) and (CAT) measurement

Serum SOD activity was estimated by Cayman’s Superoxide Dismutase Assay Kit. Tetrazolium salt was used in this assay for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine at 450 nm. SOD one unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radicals.

Cayman’s Catalase Assay Kit was used to measure serum CAT activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced is measured with 4-amino-3-hydrazone-5-mercapto-1,2,4-triazole (Purpald) as the chromogen at 540 nm.

2.10. Histopathological observation

At the end of experiment, the animals were sacrificed, and the pancreas, liver, and kidney were excised, washed with normal saline, and stored in 10% formalin. The tissue was washed, dehydrated with alcohol, and cleared with xylene, and paraffin blocks were prepared. Serial 5-μm-thick sections were cut using a rotary microtome. The sections were then deparaffinised with xylene and hydrated in descending gradients of alcohol. The slides were then transferred into haematoxylin and
for 10 min and then rinsed several times with water. These sections were examined and subsequently counterstained with eosin, rinsed with water, dehydrated with ascending gradients of alcohol, cleared with xylene, and mounted.

2.11. Immunohistochemical staining

The staining was performed as previously described (Sohn et al., 2007). The antibodies used in this study included rabbit anti-Bax (1:200, Abcam) and rabbit anti-Bcl-2 (1:250, Abcam). To detect Bax and Bcl-2, the kidney sections were incubated with the LSAB kit (DAKO K0609, CA, USA) and visualised with 3,3-diaminobenzindihydrochloride. The negative controls for immunohistochemistry were obtained by incubating the sections with non-immune serum instead of the primary antibody. For morphometric analysis, the positive cell numbers and positive signals for each tissue of a total of 40 slides were determined using the Image J software (NIH, Bethesda, MD, USA).

2.12. Statistical analysis

The statistical analysis was performed using the SPSS software package (Version 20). The data analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey’s test. All of the results were expressed as the means ± SD from the results of six rats in each group. P-values less than 0.05 were considered significant.

3. Results

3.1. Acute toxicity study

At the maximum and minimum doses of the individual and combined treatments of QE and QA, no lethal or toxic reactions were observed in the acute oral toxicity study (data not shown), which revealed their non-toxic nature.

3.2. Effects of different doses of quercetin and quinic acid on the blood glucose levels of fasting diabetic rats

In Table 1, the blood glucose levels of the treated groups are compared with those of the diabetic control group (P < 0.05), and the findings reveal that the DC group showed a significantly higher glucose level compared with the NC group. According to the percentage decrease in the blood glucose level after each treatment dose shown in parentheses for week 6, the decrease in the glucose level due to treatment was less than half of that obtained with the combined treatment. In addition, increasing the dose of QA and QE did not result in a marked decrease in the blood glucose levels compared with the combined treatment of QE + QA. Furthermore, all of the doses of QE and QA significantly decreased the blood glucose levels in diabetic rats (P < 0.05) from weeks 4 to 6. However, all of the doses of QEA + QAa produced a significant (P ≤ 0.05) decrease in the blood glucose level after week 3 and presented the highest reduction at week 6 compared with the DC and PC groups.

3.3. Effects of different doses of quercetin and quinic acid on biochemical parameters

The serum insulin, C-peptide, albumin, and total protein levels in the DC group were significantly declined (Fig. 1A–D), whereas the HbA1c levels were significantly increased compared to the NC group after the six-week treatment period (Fig. 1E). The QEA, QEB, QAa, and QAB groups showed increases in the serum insulin, C-peptide, total protein, and albumin levels, and the QAa group showed the highest increase. Similarly, the QEA, QEB, QAa, and QAB groups showed decreases in the HbA1c levels, and the QAa group showed the highest decrease. However, the diabetic rats in the QEA + QAa group displayed a significant increase in the serum insulin, C-peptide, total protein, and albumin levels, whereas the HbA1c levels significantly decreased after treatment with QEA + QAa compared with those observe in the DC rats and similar to those observed in the PC group, indicating that the combined administration of both compounds to diabetic rats resulted in a significant improvement in glycaemic control.

3.4. Effects of different doses of quercetin and quinic acid on lipid profiles

As shown in Fig. 2, the effects of the individual and combined treatments of QE and QA on the serum TC (A), LDL-C (B), and HDL-C (C) activities in STZ diabetic rats and normal control rats were observed. The serum TC and LDL-C levels in the DC group were significantly increased compared with those of the NC group (Figs. 2A and B), whereas the HDL-C levels in the DC group were significantly decreased compared with those of the NC group (Fig. 2C). Among the individual doses of QE and QA, QAa showed the highest reduction in the serum TC and LDL-C levels and similarly reflected the highest increase in the HDL-C levels. However, there was a significant reduction in the serum TC and LDL-C levels after treatment in the PC and QEA + QAa groups, whereas the HDL-C levels were significantly elevated compared with those of the DC group after the six-week treatment period. Remarkably, continuous treatment with QEA + QAa decreased these lipid parameters in diabetic rats to near-normal levels.

3.5. Effects of different doses of quercetin and quinic acid on the serum MDA level and antioxidant enzymes

3.5.1. Serum lipoperoxidation (MDA) level

As shown in Fig. 3A, DC significantly increased the serum MDA levels (0.46 ± 0.02), and this effect was gradually recovered by an individual or combined dose of QA and QE; however, the QAa group presented the lowest MDA levels compared with the other

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Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment</th>
<th>Treatment weeks</th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>5.93 ± 0.51</td>
<td></td>
<td>5.58 ± 0.42</td>
<td>5.74 ± 0.61</td>
<td>5.77 ± 0.58</td>
<td>5.67 ± 0.57</td>
<td>5.50 ± 0.38</td>
<td>5.77 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>DC</td>
<td>18.66 ± 1.51</td>
<td></td>
<td>18.74 ± 2.23</td>
<td>18.95 ± 1.92</td>
<td>20.76 ± 1.10</td>
<td>20.68 ± 0.80</td>
<td>20.70 ± 0.70</td>
<td>19.98 ± 0.45</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>19.99 ± 0.95</td>
<td></td>
<td>18.48 ± 0.82</td>
<td>16.65 ± 0.56</td>
<td>15.55 ± 0.41</td>
<td>14.74 ± 0.28</td>
<td>13.29 ± 0.40</td>
<td>11.00 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>QEa</td>
<td>19.87 ± 1.13</td>
<td></td>
<td>19.55 ± 1.81</td>
<td>19.21 ± 1.44</td>
<td>19.14 ± 1.69</td>
<td>18.27 ± 1.37</td>
<td>17.57 ± 1.28</td>
<td>16.94 ± 1.19</td>
<td></td>
</tr>
<tr>
<td>QEb</td>
<td>20.43 ± 1.65</td>
<td></td>
<td>18.69 ± 1.78</td>
<td>17.80 ± 1.44</td>
<td>17.45 ± 1.28</td>
<td>17.03 ± 1.36</td>
<td>16.75 ± 1.35</td>
<td>16.51 ± 1.07</td>
<td></td>
</tr>
<tr>
<td>QAa</td>
<td>19.5 ± 1.39</td>
<td></td>
<td>18.71 ± 1.01</td>
<td>18.46 ± 0.97</td>
<td>18.26 ± 1.01</td>
<td>17.95 ± 0.90</td>
<td>17.44 ± 0.56</td>
<td>16.85 ± 0.49</td>
<td></td>
</tr>
<tr>
<td>QAb</td>
<td>20.7 ± 1.41</td>
<td></td>
<td>19.45 ± 0.72</td>
<td>18.83 ± 0.48</td>
<td>19.99 ± 3.06</td>
<td>17.47 ± 0.49</td>
<td>17.04 ± 0.55</td>
<td>16.61 ± 0.45</td>
<td></td>
</tr>
<tr>
<td>QEA + QAa</td>
<td>18.73 ± 2.09</td>
<td></td>
<td>18.71 ± 1.81</td>
<td>17.33 ± 1.47</td>
<td>15.76 ± 0.93</td>
<td>14.21 ± 0.69</td>
<td>12.99 ± 0.68</td>
<td>11.36 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>QEB + QAb</td>
<td>19.68 ± 1.62</td>
<td></td>
<td>19.28 ± 1.59</td>
<td>18.22 ± 1.49</td>
<td>16.49 ± 0.92</td>
<td>15.47 ± 0.30</td>
<td>14.44 ± 0.69</td>
<td>13.23 ± 0.49</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD of 6 rats from each group. A Mean values revealed by the Tukey comparisons test (P < 0.05).

* Significant difference compared to DC (P < 0.05).
# Significant difference compared to NC (P < 0.05).
individual-dose treatments. The combined treatment with QEa + QAa completely recovered the increase in the MDA levels (0.27 ± 0.01) (P < 0.05) compared with the DC group.

3.5.2. Serum antioxidant enzyme activities

The serum antioxidant enzyme activities (Figs. 3B and C) demonstrated that the DC treatment significantly decreased the hepatic antioxidant enzyme activities, such as SOD and CAT (16.46 ± 0.80) (132.28 ± 1.66), compared with the NC group (P < 0.05). Treatment with QE and QA gradually increased the levels of these antioxidants, and the QAa group exhibited the highest increase among the individual treatment groups. However, QEa + QAa significantly reversed the STZ-induced decrease in the SOD and CAT activities to levels similar to those observed in the PC group (27.88 ± 0.82 and 193.98 ± 3.50, respectively) compared with the DC group (P < 0.05).

3.6. Histopathological Observation

3.6.1. Histological observations of the liver

As shown in Fig. 4, rat liver sections were stained with haematoxylin and eosin, as previously described. The magnification was 400× for all of the panels. Panel (A) shows that the NC group exhibited a normal hepatic architecture with normal hepatocyte morphology, organised hepatic cell (Hc) cords, and a portal vein (Pv) with sinusoidal cords (S). Panel (B) shows that the DC treatment resulted in severe pathological changes, such as disordered hepatic cords, focal necrosis, congestion in the portal vein, infiltration of lymphocytes, increase in Kupffer cells (Kc), and dilated sinusoids (S). Panel (C) shows the effect of the QEa treatment on the liver of a diabetic rat, and the results show the attenuation of STZ-induced hepatic cellular necrosis and fibrotic changes in the rat liver. Sinusoidal dilatation continued to be observed with decreases in the number of Kupffer cells and RBC infiltration (Rc). Panel (D) shows the effects of QEa treatment on the liver of a diabetic rat. The sinusoid remained dilated, and RBC was still observed. In addition, the central vein was presented in an orderly manner. Panel (E) shows the effect of QAa treatment on the liver of a diabetic rat, and the degeneration of the hepatocytes and RBC remained obvious. Panel (F) displays the effect of QAa treatment on the liver of a diabetic rat, and the regeneration of the hepatocytes and RBC remained obvious. Panel (G) shows the effect of the QEa + QAa combined treatment on the diabetic rat liver. The hepatic architecture was similar to the normal hepatic architecture and was characterised by a normal central vein,
normal hepatocytes, no fatty changes, and mild RBC infiltration. Panel (H) demonstrates the effect of the QEb + QAb combined treatment on the diabetic rat liver, which is characterised by a small amount of inflammatory cell infiltration. Panel (I) shows that the GL in the PC group exerted a positive effect on the STZ-induced pathological changes.

3.6.2. Histological observations of the pancreas

As shown in Fig. 5, the staining of the rat pancreas sections with haematoxylin and eosin was performed as previously described in the methods section. In panel (A), the NC pancreatic section showed normal islets of Langerhans, a normal pancreatic structure, and normal acini tissues. Panel (B), which corresponds to the DC group, shows a disorganisation of the structure of the endocrine and exocrine cells with damaged and necrotic pancreatic acini and islet shrinkage. In panel (C), the QEa treatment exerted minimal restoration of pancreatic endocrine cells and expansion of pancreatic islets, which showed a prominent hyperplastic islet. As shown in Panel (D), QEb treatment resulted in a disorganised cell structure, and the degeneration of some of the pancreatic acini was still observed. Panel (E) shows the effect of QAa treatment on the diabetic rat pancreas, which is shown in Panel (H), exhibits a moderate restoration of pancreatic cells. As shown in Panel (I), the PC groups exhibited the most interesting aspect of the examined pancreatic sections treated with positive control drug, Glibenclamide which showed normal islets of Langerhans.

3.6.3. Histological observations of the kidney

As shown in Fig. 6, the rat kidney sections were stained with haematoxylin and eosin as described in the methods section. The

Fig. 2. Effects of quercetin and quinic acid with different doses on serum lipid profiles of STZ-induced diabetic rats with DC after the 6-week treatment period. At the end of the treatment, rats were fasted for 12 h and blood was drawn to collect the serum. Figure represents TC (A), LDL-C (B), and HDL-C (C), levels in 9 groups. Data are presented as means ± SD (n = 6). (*) Significant difference compared to DC (P < 0.05). (#) Significant difference compared to NC (P < 0.05).

Fig. 3. Effects of QA & QE and combined administration of QA + QE in different doses on the serum MDA (A), SOD (B) and CAT (C) levels of in the STZ-induced rats. Each bar was represented as mean ± SD (n = 6). (*) Significant difference compared to QA + QE (P < 0.05), compared with DC. (#) Significant difference compared to NC (P < 0.05).
magnification was 400× for all of the panels. As shown in Panel (A), the NC rat kidney presents a normal glomerulus surrounded by Bowman’s capsule, proximal and distal convoluted tubules without any inflammatory changes, normal capillaries (CP), and normal podocytes (Pc). Panel (B) shows the kidneys of the DC-treated rats, which present degenerated glomeruli infiltrated by inflammatory cells and a thickening of the basement membrane. The proximal convoluted tubule exhibited oedematous changes with a deposition of mucopolysaccharide and hyaline substances. Furthermore, it exhibited severe pathological damages, such as mesangial expansion and glomerular hypertrophy (Gh). Panel (C) shows the effect of QEa treatment, and the findings present features of healing, i.e., less expansion of the glomerulus, the presence of few inflammatory cells, mildly dilated capillaries, and decreased mucopolysaccharide and hyaline deposits. Panel (D) shows that the QEb treatment results in less changed morphology and a decreased expansion of the glomerular and mesangial matrix. Panel (E) shows the effect of QAa treatment on the diabetic rat liver with moderate changes in the kidney. As shown in Panel (G), the analysis of the renal histology after the QEa + QAa combined treatment showed...
Fig. 5. H & E staining of different group of rats pancreas (400×1). Panel (A): NC (B) DC (C) QEa (D) QEb (E) QAa (F) QAb (G) QEa + QAa (H) QEb + QAb (I) PC. In panel (A), the NC pancreatic section showed normal islets of Langerhans. Panel (B) DC shows a disorganisation of the structure of the endocrine and exocrine cells with damaged and necrotic pancreatic acini and islet shrinkage. In Panel (G), the effect of QEa + QAa on diabetic rats liver demonstrates that these factors better promoted the cells and provides a more healthy structure and shows a prominent hyperplastic islet and improved islet morphology. Treatment of QEb + QAb in Panel (H) exhibited moderate restoration of pancreatic cells on the diabetic rat. The treatment of positive control (Glibenclamide) in Panel (I), demonstrated the most interesting aspect of the examined pancreatic sections, which showed normal islets of Langerhans.
no clear histopathological abnormalities and a decreased expansion of the glomerular and mesangial matrix. Panel (I) shows the PC group. These broad changes caused the loading of the Bowman’s capsule space and an adhesion of the capillaries to the wall.

Fig. 6. H & E staining of different group of rats kidney (400×). Panel (A): NC (B) DC (C) QEa (D) QEb (E) QAa (F) QAb (G) QEa + QAa (H) QEb + QAb (I) PC. In diabetic rats (B), there is a significant damage to the glomeruli (hypertrophy, hypercellularity, tubules dilatation and atrophy) compared to the normal control (A). Treatment of QE and QA attenuated the kidney alteration (C,D,E,F,G,H) in diabetic rats, which are comparable with the positive control (I) on the STZ-induced pathological changes.
3.7. Immunohistochemistry evaluation

3.7.1. Effect of quercetin and quinic acid on the Bcl-2 expression level in the renal glomeruli

The Bcl-2 protein expression in the kidney tissues was qualitatively analysed using immunohistochemistry, as shown in Fig. 7A, and the findings show that the level of Bcl-2 protein expression was markedly reduced in the DC group compared with the NC group. However, this decrease in the Bcl-2 level was gradually recovered after the administration of different doses of QE and QA. The arrows indicating QAa and QAb show an increase in protein expression compared with QEa or QEb. However, the QEa + QAa combined treatment (Panel G) exhibited a significant increase in Bcl-2 protein expression compared with the DC and PC groups (Panel I). The histological results were confirmed using a quantitative and histomorphometry study, as shown in Fig. 7B, which shows that the DC group (0.002 ± 0.001) exhibits a significant decrease in Bcl-2 expression compared with the NC group (0.035 ± 0.001). However, different doses of QE and QA significantly enhanced the expression of Bcl2 compared with the DC group (P < 0.05), and the QEa + QAa group displayed the highest increase in the expression level of this protein (0.032 ± 0.001).

3.7.2. Effect of quercetin and quinic acid on Bax expression level in renal glomeruli

The Bax protein expression in the kidney tissues was qualitatively analysed using immunohistochemistry. As shown in Fig. 8(A), the level of Bax protein expression was increased in the DC group compared with the NC group, and this increase was gradually recovered with different individual doses of QE and QA. The comparison of the individual treatment groups revealed that the QAa and QAb groups showed a higher reduction in the Bax expression levels compared with the QEa and QEb groups, as shown by the two arrows in each panel. No arrows are shown in Fig. 8(A) G, which indicates that the QEa + QAa treatment significantly decreased Bax protein expression compared with the DC and PC groups. These histological results were confirmed using a histomorphometry study, which revealed a significant increase in Bax expression in the DC group (0.041 ± 0.001) compared with the NC group (0.001 ± 0.001) (Fig. 8B). Furthermore, different doses of QE and QA can significantly inhibit the expression of Bcl2 compared with the DC treatment. However, Bax expression was more significantly reduced by the combined administration of QEa + QAa (0.002 ± 0.001) compared with the DC treatment.

4. Discussion

Flavonoids are a group of naturally occurring compounds that are widely distributed as secondary metabolites in the plant kingdom. It has been demonstrated that these compounds have interesting clinical properties, such as anti-inflammatory, anti-allergic, antiviral, antibacterial, and antimiturial activities (Middleton, 1998). One of these flavonoids, namely quercetin (3,5,7,3,4-pentahydroxyflavone), can prevent oxidative injury and cell death by several mechanisms, such as the scavenging of oxygen radicals (Inal et al., 2002), which protect against lipid peroxidation (Middleton, 1998). Another polyphenol-rich compound is quinic acid, which has been previously reported to be an active compound with significant antioxidant and anti-hyperglycaemic properties (Pero et al., 2009). In addition, this compound is capable of reducing the oxidative stress burden in cells (Saltiel and Kahn, 2001). Because there is no scientific evidence of the combined effect of quercetin and quinic acid on experimental diabetic rats, this study aimed to investigate the synergistic anti-hyperglycaemic activity of these two flavonoids on STZ-induced diabetic rats.

Our study demonstrated no signs of toxicity with QE or QA. The National Toxicology Program performed a renal toxicity study of quercetin and found an increase in the severity of chronic nephropathy, hyperplasia, and neoplasia of the renal epithelium in male rats treated with 2000 mg/kg of body weight, but no adverse effects were observed with lower doses of 50 and 500 mg/kg of body weight (Aguirre et al., 2011). According to our results, the combined administration of QEa + QAa suggested a marked improvement in hyperglycaemia and hyperlipidaemia after four weeks of administration. The decrease in the elevated blood glucose in the DC group and the normalisation of the glucose levels in the QEa + QAa group are shown in Table 1, and these results may be due to the stimulatory activity of quercetin and quinic acid, both of which have nearly no effect on the glycaemic status and oxidative stress markers in normal rats but have significant effects on the glucose level parameters in STZ-treated rats (Arya et al., 2012a; Prince and Kamalakkannan, 2006). The hypoglycaemic effect of both compounds may be due to their antioxidant properties (Sanders et al., 2001; Pero et al., 2009; Arya et al., 2012b; Taha et al., 2014).

Until recently, insulin was the sole pancreatic β-cell hormone that is known to lower blood glucose levels (Brezar et al., 2011). Healthy individuals sustain stable blood glucose levels through basal insulin secretion (Raghavan and Garber, 2008), but hyperglycaemia in diabetes is the main symptom caused by a lack of insulin and/or insulin resistance (Ke et al., 2009). Consistent with these findings, we showed reduced insulin levels in the DC group, but this reduction was restored by treatment with QEa + QAa to a level similar to that observed in the PC group (Fig. 1A). In addition, Vessal et al. (2003) suggested that quercetin supplementation is beneficial for decreasing the blood glucose concentration, thereby promoting the regeneration of pancreatic islets and increasing insulin release in STZ-induced diabetic rats and thus exerting its beneficial anti-diabetic effects. The most reliable primary outcome of the investigation of diabetes treatment is the C-peptide levels, which further validated the assessment of β-cell function and aimed to preserve or improve endogenous insulin secretion (Palmer et al., 2004). The serum C-peptide levels were significantly increased in the QEa + QAa group, which is consistent with the results reported by Taha et al. (2014), and may be due to the increased secretion of insulin from remnant β-cells (Fig. 1B).

As shown in Fig. 1C, the level of albumin was markedly reduced in the DC group, which may be due to impaired tubular reabsorption or leakage of albumin due to glomerular basement membrane damage combined with an increase in transglomerular filtration pressure. However, the QEa + QAa combined treatment significantly increased the serum albumin towards the NC and PC levels, which was reflected in the reduction of kidney damage due to STZ-induced hyperglycaemia. Consistent with our results, Kandasamy and Ashokkumar (2012) concluded that flavonoids restore the reduced albumin level in diabetic nephrotoxic rats. Thus, QE and QA, particularly at a dose of 50 mg/kg, demonstrate a beneficial pharmacological effect on diabetic, hepatic, renal functional markers and protein levels. Persistent hyperglycaemia results in the glycation of Hb, which results in the formation of HbA1C (Yabe-Nishimura, 1998). Furthermore, the QEa + QAa combined treatment significantly decreased the levels of HbA1C and increased the level of serum insulin in experimental diabetic rats. Agents with antioxidant or free radical-scavenging properties have been shown to inhibit oxidative reactions associated with glycation (Elgawish et al., 1996). Hill and Howell (1984) reported that the exposure of isolated rat islets to specific flavonoids, such as quercetin, enhances insulin release by 44–70%.
Fig. 7. (A) Effect of quercetin and quinic acid on Bcl-2 expression level in the diabetic renal rat. Immunohistochemical staining (400×). Panel (A): NC (B) DC (C) QEa (D) QEb (E) QAa (F) QAb (G) QEa + QAa (H) QEb + QAb (I) PC (Bcl-2 expression shown with arrows). (B) Representative immunohistochemical staining and quantitative analysis of different doses of quercetin and quinic acid treatment on Bcl-2 expression showed in kidney of diabetic rats. Data were analysed by Image analysis software as mean ± SD by one-way ANOVA (Tukey). (*) indicated $P < 0.05$ different from DC ($n = 6$ per group). (#) Significant difference compared to NC ($P < 0.05$).
Fig. 8. (A) Effect of quercetin and quinic acid on Bax expression level in the diabetic renal rat. Immunohistochemical staining (400×). Panel (A): NC (B) DC (C) QEa (D) QEb (E) QAa (F) QAb (G) QEa + QAa (H) QEb + QAb (I) PC. (Bax expression shown with arrows). (B) Representative immunohistochemical staining and quantitative analysis of different doses of quercetin and quinic acid treatment on Bax (B) expression showed in kidney diabetic rats. Data were analysed by Image analysis software as mean ± SD by one-way ANOVA (Tukey), (*) indicated $P < 0.05$ different from DC ($n = 6$ per group). (#) Significant difference compared to NC ($P < 0.05$).
According to the data shown in Fig. 2A and B, the levels of serum TC and LDL-C were significantly elevated in the DC group, whereas the levels of HDL-C were decreased (Fig. 2C). Thus, the successive combined administration of QEa + QAa produced an obvious reduction in the serum TC and LDL-C levels and an increase in HDL-C, which is consistent with the findings reported by Kandhare et al. (2012). Furthermore, the flavonoid-rich compound quercetin exerted a significant reduction in the serum cholesterol and LDL concentrations in alloxan-diabetic rats (Nuraliev and Avezov, 1991). In addition, the QEa + QAa combined treatment attenuated the disorder of lipid metabolism, similarly to the effect observed in the study conducted by Wang et al. (2012).

It has been pre-clinically and clinically proven that the level of lipid peroxidation is significantly increased in the plasma of diabetic rats and diabetic patients (Sato et al., 1979). Furthermore, lipid peroxidation, which is a free radical-mediated oxidation of polyunsaturated fatty acids, is increased in the DC group, as is evident from the elevated MDA levels. The QEa + QAa combined treatment effectively reverts the serum MDA to normal levels (Fig. 3A), suggesting the antioxidant functions of the combination of these flavonoids, which is comparable to the findings reported by Machha et al. (2007). In addition, the imbalance between the formation of reactive oxygen species, i.e., free radicals, and the endogenous antioxidant enzymes results in the generation of oxidative stress ( Cotter and Cameron, 2003). SOD is responsible for the conversion of superoxide anions to H2O2 and thus reduces oxidative stress. In our results, the QEa + QAa combined treatment efficiently restored the depleted status of the antioxidant enzymes SOD and CAT (Fig. 3B and C), indicating the combined antioxidative properties of the two polyphenols. The results of the present investigation are consistent with the findings reported by Arya et al. (2012c) and Dias et al. (2005).

In our histopathology study, the DC group showed many pathological changes, such as degenerated hepatocytes with polymorphic nuclei, focal necrosis, congestion in the portal vein, infiltration of lymphocytes, increases in Kupffer cells, dilated sinusoids, and mononuclear cell infiltrates extending throughout the hepatic tissue, which is similar to the effects observed in previous diabetic animal models (Motshakeri et al., 2014). The unorganised hepatocytes, microvascular vacuolisation, granular degeneration, and necrosis are marked changes observed in the diabetic liver (Zhou et al., 2008). Previous histological studies have shown that quercetin rescued the liver damage of diabetic rats by increasing the activity of antioxidant enzymes and by scavenging hydroxyl, superoxide, alkoxyl, and peroxy radicals (Kandasamy and Ashokkumar, 2012). Consistent with our findings, the severity of hepatic injuries were reduced with the QA and QE individual treatments and with the QEa + QAa combined treatment; no considerable fatty changes were observed, indicating the protective effect of these two flavonoids against the hepatic complications of diabetes, as shown in Fig. 4.

Roat et al. (2014) suggested that any alteration to the structure, size, and function of islets indicates metabolic changes related to insulin secretion, insulin sensitivity, and loss of glycaemic control. In the study conducted by Motshakeri et al. (2014), the STZ-induced diabetic SD rats presented small islets, including degenerated cells and reduced β cells, which is consistent with the findings obtained by Roat et al. (2014). Similarly, the pancreas tissues of diabetic rats revealed a reduction in the number of islets, degeneration of β cells, hydropic degeneration, clumping of β cells, pyknosis, and necrosis, which caused a change in the morphology of the cells due to the STZ-induced partial damage of some β cells (Fig. 5). In the present study, the islet cells were restored by QE and QA, which recovered the degenerated cells. This result was consistent with those obtained by Rifaai, who reported that quercetin improves degenerated cells with no inflammatory cell infiltration (Rifaa et al., 2012). However, our studies showed that the administration of an increased individual dose of QEb and QAa to diabetic animal may cause reversible effects, unlike the combined administration of QEa + QAa, suggesting that there were more functional cells in the QEa + QAa group compared with the individual treatment groups the group with the higher combination dose (QEb + QAab). This finding may explain the higher level of insulin that was found (Ong et al., 2011). In addition, the QE and QA treatments restore the pancreas histology by alleviating oxidative stress, which is in tune with the results reported by Fernandez-Alvarez et al. (2004).

One of the major complications associated with diabetes is diabetic nephropathy (DN), which results from the expansion of mesangial cells, a hallmark of diabetic rats (Matsubara et al., 2006), the accumulation of extracellular matrix protein, the thickening of the glomerular and tubular basement membranes, tubulointerstitial fibrosis, glomerulosclerosis, renal endothelial dysfunction, albuminuria, proteinuria, and a reduction in the glomerular filtration rate (Balakumar et al., 2009). In Fig. 6, the DC group showed acute swelling of cells, hydropic degeneration of tubules, widening of Bowman’s space, glomerular atrophy, congestion of capillaries, and tubular necrosis. The severe pathological changes observed involved focal mesangial matrix expansion, proteinuria, and a significant increase in albuminuria compared with the NC group. A reduced degree of expansion and degeneration was observed from Panel C to H compared to Panel I (the QE and QA treatments), which is consistent with the results obtained by Bashir et al. (2014), who showed that the quercetin-treated diabetic rats demonstrated a recovery of the normal kidney structure with intact glomerular epithelial cells and tubules. However, the QEa + QAa combined treatment ameliorated the mesangial expansion, proteinuria, and albuminuria, and this dosage combination was the most beneficial dose for alleviating histological injuries compared with the other doses.

Cell apoptosis is directly affected by multiple genes inside cells. Among these genes, the most representative are the pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) genes (Lian et al., 2011). In this study, we found that the QEa + QAa combined treatment ameliorated the increased pro-apoptotic Bax protein level and the decreased Bcl-2 protein level in the accumulated glomeruli of STZ-induced diabetic rats. Bcl-2 proteins play an important role in β-cell physiology and apoptosis due to their anti-apoptotic properties. Thus, the presence of these genes can result in the increased survival of β cells (Gurzov and Eizirik, 2011). According to Li et al. (2005), an upregulated expression of Bcl-2 proteins is induced by treatment with high glucose, which suppresses apoptosis in VSMCs. Diabetes is the result of reduced insulin secretion by β cells and thus reduced expression of Bcl-2 genes, as observed in the DC group. The decreased Bcl-2 expression was completely restored by the QEa + QAa combined treatment, which is comparable to the results obtained by Gurzov and Eizirik (2011). However, Bax is pro-apoptotic, which indicates that it promotes the apoptosis of β cells and exhibits increased expression in diabetes, but its expression is downregulated by treatment with QEa + QAa. Our results, which were confirmed through a quantitative analysis (Fig. 8A and B), were consistent with previous reports that showed that diabetic kidney cells undergo apoptosis both in vitro and in vivo via the activation of Bax protein and downstream targets, which support the treatment of diabetes via the prevention of apoptosis (Eslami and Sharifi, 2012). It has been reported that the QEa + QAa combined treatment demonstrates a protective effect against cell apoptosis (Sohn et al., 2010). The accumulation of QEa + QAa in the kidney and their anti-apoptotic effects on podocytes may ameliorate diabetic effects. As a result, the combined flavonoid approach is an effective treatment for diabetes in the kidney and may be a valuable therapeutic approach.
Taken together, our study revealed the hypoglycaemic activity of quercetin and quinic acid, but the precise mechanism underlying this phenomenon remains unclear. However, it can be assumed that the two promising phytochemicals, quercetin and quinic acid, protect pancreatic β-cells due to their antioxidant properties, as evidenced by an increase in antioxidant status and a decrease in lipid peroxidation, which resulted in insulin release in the diabetic-treated rats and may reduce the risk of diabetic complications. Similar results have been reported with flavonoids, such as quercetin and rutin, which showed nearly no effect on the glycemic status and oxidative stress markers in normal rats but have significant effects on antioxidant parameters in STZ-treated rats (Prince and Kamalakannan, 2006). However, our results for the combined low dose of quercetin and quinic acid showed the maximum protective effect compared with the individual dose treatments and the higher-dose combination therapy.

5. Conclusions

In summary, the combined administration of QEa + QAa exerted an anti-hyperglycaemic effect in a STZ-induced diabetic rat model. Consistent with previous studies describing increased insulin secretion by flavonoids, we propose that the increase in insulin levels induced by treatment with QEa + QAa is due to the protective effect of quercetin and quinic acid on the liver, kidney, and pancreatic β-cells. Furthermore, the QEa + QAa combined treatment also showed a positive modulation of the lipid profile, antioxidants, and Bcl-2 defence. Taken together, these biological effects may be attributed to the combinatorial effects of both the compounds. Thus, further studies are warranted to investigate the deep mechanism and synergistic effects of the flavonoids to create possibility. Further studies are warranted to investigate the deep mechanism and synergistic effects of the flavonoids to create possibility.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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