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SUMMARY

This study was conducted to evaluate the efficacy of mulberry (MLE) and jackfruit (JLE) leaf extracts, alone or in combination, compared with gliclazide, on streptozotocin-nicotinamide-induced diabetic rats for 8 weeks. Results showed significant increase in plasma glucose, HbA1c %, TBARS, NOx and liver tissue DNA damage levels in diabetic rats compared with normal controls, whereas liver glycogen, GSH levels and erythrocyte SOD and CAT activities were significantly decreased in the former. Plasma glucose levels and HbA1c % were significantly decreased by treatment with MLE or JLE, and normalized by gliclazide. Alterations in plasma TBARS, GSH and NOx levels were reverted to near normal with MLE and a mixture. Moreover, the mixture restored the activities of erythrocyte SOD and CAT, while JLE restored GSH and CAT to normal values. All treatments resulted in disappearance of DNA damage in diabetic livers. Finally, we concluded that MLE or JLE treatment exerted a therapeutic protective effect on diabetes.

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (1). In 2013, an astounding 382 million people were estimated to have diabetes, with dramatic increases seen in countries all over the world. If current demographic patterns continue, more than 592 million people will be affected with diabetes within a generation (2).

Despite the availability of many antidiabetic medicines on the market, diabetes and related complications continue to be major medical problems (3). The use of these drugs is accompanied with their side effects (4). The search for more effective and safer hypoglycemic agents continues to be an important area of research (3).

Some medicinal plants have been reported to be useful in diabetes worldwide and have been used empirically as antidiabetic and antihyperlipidemic
remedies (5). The medicinal plants may provide a useful source of new oral hypoglycemic compounds for the development of pharmaceutical entities or as dietary adjunct to existing therapy (6). Of these medicinal plants widely used in the field of herbal medicine, *Morus alba* and *Artocarpus heterophyllus* have been reported to possess several medicinal properties including hypoglycemic effect (7-10).

*Morus alba*, known as mulberry, has long been used as an antiphlogistic, diuretic, antihyperlipidemic and antidiabetic remedy in traditional medicine (3). A toxicity study of *Morus alba* leaves revealed no remarkable acute or subacute toxicities (11). Mulberry water extracts contain polyphenols, including gallic acid, chlorogenic acid, rutin and anthocyanins (12). Furthermore, the pipperidine alkaloid and glycoproteins from the *Morus* root bark and/or leaf extract have been used as antidiabetic agents (4).

*Artocarpus heterophyllus*, commonly known as jak, is reported in Ayurveda to possess antibacterial, anti-inflammatory, antidiabetic, antioxidant and immunomodulatory properties. It was found to be useful in fever, boils, wounds, skin diseases, convulsions, diuresis, constipation, ophthalmic disorders, snake bite, etc. (13). The presence of phytoconstituents like phenolics, sterols, triterpenoids and carbohydrates indicates that the plant can be useful for treating different diseases (13). In previous studies, it was reported that aqueous extract of *Artocarpus heterophyllus* leaves possessed a significant oral hypoglycemic activity and was believed to improve glucose tolerance in normal subjects and diabetic patients (14). Studies performed by Chandrika et al. (15) showed the flavonoid fraction to have the highest hypoglycemic activity.

The present work aimed to study the possible ameliorative effects of *Morus alba* and *Artocarpus heterophyllus* on experimentally induced type 2 DM comparing their effects, alone or in combination, with a commercially known drug, gliclazide, as a reference agent.

**MATERIALS AND METHODS**

**Animals**

The animal studies were implemented in compliance with the policies outlined in the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Experiments were carried out using adult female Sprague-Dawley rats, weighing 150-180 g, obtained from the animal house of National Organization for Drug Control and Research (NODCAR), Giza, Egypt. Rats were housed in groups of five per cage under controlled environmental conditions of temperature and humidity and exposed to a 12-h light/dark cycle. Unless otherwise indicated, animals were fed on normal laboratory chow and were given tap water *ad libitum* throughout the experimental period.

**Induction of experimental diabetes**

Experimental type 2 DM was induced in overnight fasted rats using a single intraperitoneal (i.p.) injection of streptozotocin (STZ) (Sigma, St. Louis, MO, USA; 65 mg/kg b.w.) dissolved in 0.09 M citrate buffer (pH 4.5), immediately before use, 15 min after the i.p. administration of nicotinamide (NA) (16, 17) (Fluka Chemie AG, Buchs, Switzerland; 110 mg/kg b.w.) with a modification in NA dose (18). Hyperglycemia was confirmed by the elevated fasting plasma glucose (FPG) levels, determined on the 3rd and 7th day after injection of STZ/NA. Only rats that were found to have moderate and stable hyperglycemia (170-250 mg/dL) were considered diabetic and involved in the experiment.

**Experimental design**

The animals were randomized into six groups as follows: I) normal non-diabetic control (N) group; II) untreated diabetic (D) group; both received 1% Tween 80 in distilled water as a vehicle (10 mL/kg b.w.); III) mulberry leaf extract-treated diabetic group (D+MLE); IV) jackfruit leaf extract-treated diabetic group (D+JLE); V) mixture-treated diabetic group (D+MLE+JLE); VI) gliclazide-treated diabetic group (D+G).

The animals were housed under controlled environmental conditions of temperature and humidity and exposed to a 12-h light/dark cycle. Unless otherwise indicated, animals were fed on normal laboratory chow and were given tap water *ad libitum* throughout the experimental period.
(D+MLE+JLE): received MLE, JLE or an equimixture of MLE and JLE (500 mg/kg b.w.) dissolved in 1% Tween 80 in distilled water as a vehicle, respectively. Each mL of all the previous extracts was standardized to contain 50 mg extract; and VI) gliclazide-treated diabetic group (D+GLZ): received gliclazide (14.4 mg/kg b.w.) suspended in 1% Tween 80 in distilled water as a vehicle.

All treatments were given orally for 8 weeks by gavage and the animals were monitored through periodic testing of fasting blood glucose and body weight weekly.

Materials

Gliclazide was kindly supplied as a raw material by the Egyptian International Pharmaceutical Industries Company (EIPICO), Egypt. A concentration of 14.4 mg gliclazide/kg b.w. of rat was used, which is equivalent to 80 mg/b.w. therapeutic dose in human (19).

Chemicals for which the source is not noted were of analytical grade from Sigma, Sigma-Aldrich, Fluka, BDH, Park, Riedel-de Haën and MP Biomedicals.

Plant material collection

White mulberry leaves were collected from Morus alba tree grown in the agricultural land of the Applied Research Centre of Medicinal Plants (ARCMP), NODCAR, Giza, Egypt. Jackfruit leaves were supplied from Al-Zohrea garden (Cairo, Egypt). The plants were kindly identified as: Morus alba L. (Moraceae) and Artocarpus heterophyllus Lam. (Moraceae), respectively, and deposited in Cairo University Herbarium by Dr. Wafaa M. Amer, Botany and Microbiology Department, Faculty of Science, Cairo University, Giza, Egypt.

Preparation of aqueous plant extracts

Fresh leaves of both plants were washed and wiped, air-dried, coarsely powdered using cutter mill, added to boiling double-distilled water and allowed to infuse. The extract was decanted, filtered under vacuum, concentrated in rotary evaporator (Butchi Rotavapor R-205, Switzerland) and then lyophilized (Lyphilyzer Sniffers Scientific b.v., Tilburg, Holland) (20).

The air-dried, powdered leaves of both plants were subjected to preliminary phytochemical screening. The results revealed the presence of carbohydrates, tannins, glycosides, alkaloids and flavonoids in mulberry leaves and the presence of carbohydrates, flavonoids, glycosides, sterols, and tannins in jackfruit leaves.

Spectrophotometric determination of total polyphenolics and total flavonoids in MLE and JLE

Total polyphenolics content was colorimetrically assayed according to the method of Aliyu et al. (21) using gallic acid as a standard. The concentration of total phenolics was calculated with reference to a pre-established standard calibration curve. The method used to assay total flavonoids was that of Makky et al. (22). The concentration of flavonoids was calculated using the equation of quercetin standard curve.

All spectrophotometric measurements in the current study were performed using the UV-visible spectrophotometer (Thermo-Nicolet evolution 100, England).

HPLC assay of flavonoids and phenolic compounds in MLE and JLE

Flavonoids and phenolic compounds were HPLC-assayed according to the method of Mattila et al. (23), using a Hewlett Packard (series 1050) equipped with autosampling injector, solvent degasser, ultraviolet (UV) detector (set at 280 nm for phenolic determination and 330 nm for flavonoid determination) and quaternary HP pump (series 1050). The column temperature was maintained at room temperature. Separation was carried out using methanol and acetonitrile (2:1) as a mobile phase at a flow rate of 1 mL/min. Authentic phenolics and flavonoids (Sigma Co.) were dissolved in mobile phase and injected into
HPLC. The retention time and the peak area were used to calculate the phenolic and flavonoid concentrations by data analysis using the Hewlett Packard software.

**Determination of LD\textsubscript{50} of MLE and JLE (acute oral toxicity test)**

Acute oral toxicity studies for both extracts and their mixture were carried out as per Organization for Economic Co-operation and Development (OECD) Test Guideline 425. Mortality in each group within 24 h was recorded. The animals were further observed for another 14 days for any signs of delayed toxicity. Therapeutic dose was calculated using the equation: therapeutic dose = LD\textsubscript{50}/10 (24).

**Sample collection**

At the end of the experiment, all animals from each group were fasted overnight and blood was collected according to the procedure described by Schermer (25). Then, animals from each group were sacrificed by decapitation; livers were removed for glycogen assessment and analysis of total genomic DNA damage.

**Biochemical assays**

**Estimation of markers of diabetes**

Glucose was determined in plasma according to the enzymatic colorimetric method of Trinder (26) using Audit Diagnostics kit (Cork, Ireland). Glycosylated hemoglobin (HbA1c %) was determined in the hemolysate using the Biosystems assay kit (Barcelona, Spain), where the labile fraction is eliminated; hemoglobins are retained by a cationic change resin. HbA1c is specifically eluted after washing away the hemoglobin A1a+b fraction (Hb A1a+b), and quantified by direct photometric reading at 415 nm (27). The method used in determination of liver glycogen is that of Montgomery (28).

**Estimation of biomarkers of lipid peroxidation and oxidative stress and antioxidant enzymes**

In plasma, the extent of lipid peroxidation was estimated as the concentration of thiobarbituric acid reactive substances (TBARS) using the method of Buege and Aust (29). In erythrocytes, reduced glutathione (GSH) concentration, Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and catalase (CAT) activities were determined using the method described by Beutler et al. (30), Marklund and Marklund (31) and Aebi (32), respectively, whereas serum nitric oxide (NOx) was determined using the method of Miranda et al. (33).

**Evaluation of the antimutagenic activity of the tested plants by DNA fragmentation assay**

The method of DNA fragmentation was performed according to the salting out extraction technique of Aljanabi and Martinez (34). The intensity of DNA bands was measured using the Biogene software (Biogene Software, France).

**Statistical analysis**

Biochemical results were expressed as mean ± standard error (SE) and statistically analyzed using one-way ANOVA Statistical Package for the Social Sciences (SPSS) version 17.0 according to Snedecor and Cochran (35), followed by Tukey’s post-hoc test for multiple comparisons. Differences were considered to be statistically significant when p<0.05. Data were graphically presented using Microsoft Excel (2007).

**RESULTS**

**Spectrophotometric determination of total polyphenolics and total flavonoids of MLE and JLE**

From the standard calibration curve, the total phenolic content was calculated as gallic acid: 3.06 and 1.83 mg/100mg dry extract in MLE and JLE,
respectively, whereas, total flavonoids were calculated as quercetin: 0.409 and 0.46 mg/100 mg dry extract in MLE and JLE, respectively.

**HPLC assay of flavonoids and phenolic compounds in MLE and JLE**

As presented in Table 1, the flavonoids identified in MLE that showed the highest concentrations were hisperidin: 73.56 mg/100 g dry extract and rutin: 28.29 mg/100 g dry extract, while in JLE the highest concentrations were shown by vitexin: 163.23 mg/100 g dry extract, hisperid: 26.74 mg/100 g dry extract and quercetin: 25.35 mg/100 g dry extract. Table 1 also shows that the lowest flavonoid concentration identified in MLE was that of narengenin: 0.46 mg/100 g dry extract, while in JLE, 7-OH flavone: 0.65 mg/100 g dry extract showed the lowest concentration.

Table 2 shows that the highest concentrations of phenolic compounds identified in MLE were those of e-vanillic acid: 537.83 mg/100 g dry extract, 3-OH-tyrosol: 150.41 mg/100 g dry extract and benzoic acid: 123.31 mg/100 g dry extract, while in JLE the highest phenolic compound concentrations were shown by chicoric acid: 161.12 mg/100 g dry extract, e-vanillic acid: 133.93 mg/100 g dry extract and protochatcuic acid: 128.76 mg/100 g dry extract. Table 2 also reveals that the lowest phenolic compound concentration identified in MLE was that of gallic acid: 2.98 mg/100 g dry extract, while in JLE cinnamic acid: 3.42 mg/100 g dry extract, showed the lowest concentration. Vanillic acid was not identified in JLE but was identified in MLE: 9.22 mg/100 g dry extract.

**Determination of LD<sub>50</sub> and therapeutic dose calculation**

After administering the extracts orally to the rats in different gradual doses (1 to 5 g/kg b.w.), the animals of the three groups showed no mortality or any toxic symptoms during the first 24 hours or on the days after for as long as 2 weeks.

Table 1. Concentrations of flavonoids identified in mulberry (MLE) and jackfruit (JLE) leaf extracts using HPLC

<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>MLE (mg/100 g)</th>
<th>JLE (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitexin</td>
<td>2.54</td>
<td>163.23</td>
</tr>
<tr>
<td>Naringin</td>
<td>9.04</td>
<td>11.80</td>
</tr>
<tr>
<td>Rutin</td>
<td>28.29</td>
<td>9.17</td>
</tr>
<tr>
<td>Hisperidin</td>
<td>73.56</td>
<td>7.10</td>
</tr>
<tr>
<td>Rosmarinic</td>
<td>10.70</td>
<td>2.27</td>
</tr>
<tr>
<td>Quercetin</td>
<td>9.92</td>
<td>25.35</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.86</td>
<td>3.76</td>
</tr>
<tr>
<td>Narengenin</td>
<td>0.46</td>
<td>1.80</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.81</td>
<td>1.35</td>
</tr>
<tr>
<td>Hischeretin</td>
<td>3.77</td>
<td>26.74</td>
</tr>
<tr>
<td>Apegenin</td>
<td>0.69</td>
<td>7.42</td>
</tr>
<tr>
<td>7-OH flavone</td>
<td>0.51</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Table 2. Concentrations of phenolic compounds identified in mulberry (MLE) and jackfruit (JLE) leaf extracts using HPLC

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>MLE (mg/100 g)</th>
<th>JLE (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic</td>
<td>2.98</td>
<td>5.01</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>92.26</td>
<td>91.90</td>
</tr>
<tr>
<td>3-OH-tyrosol</td>
<td>150.41</td>
<td>23.44</td>
</tr>
<tr>
<td>4-Amino-benzoic</td>
<td>4.08</td>
<td>10.99</td>
</tr>
<tr>
<td>Protochatcuic</td>
<td>51.92</td>
<td>128.76</td>
</tr>
<tr>
<td>Chlorogenic</td>
<td>27.48</td>
<td>25.74</td>
</tr>
<tr>
<td>Catechol</td>
<td>60.26</td>
<td>60.74</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>26.28</td>
<td>27.81</td>
</tr>
<tr>
<td>Catechin</td>
<td>17.54</td>
<td>14.15</td>
</tr>
<tr>
<td>P-OH-benzoic</td>
<td>22.97</td>
<td>29.76</td>
</tr>
<tr>
<td>Caffeic</td>
<td>18.21</td>
<td>36.38</td>
</tr>
<tr>
<td>Chicoric</td>
<td>59.45</td>
<td>161.12</td>
</tr>
<tr>
<td>Ferulic</td>
<td>12.44</td>
<td>30.83</td>
</tr>
<tr>
<td>Iso-ferulic</td>
<td>6.53</td>
<td>6.31</td>
</tr>
<tr>
<td>e-Vanillic</td>
<td>537.83</td>
<td>133.93</td>
</tr>
<tr>
<td>Reversetrol</td>
<td>8.82</td>
<td>3.56</td>
</tr>
<tr>
<td>Ellagic</td>
<td>29.58</td>
<td>33.04</td>
</tr>
<tr>
<td>Alpha-coumaric</td>
<td>19.64</td>
<td>6.37</td>
</tr>
<tr>
<td>Benzoic</td>
<td>123.31</td>
<td>62.47</td>
</tr>
<tr>
<td>3,4,5-Methoxy-cinnamic</td>
<td>8.26</td>
<td>19.74</td>
</tr>
<tr>
<td>Coumarin</td>
<td>10.20</td>
<td>5.44</td>
</tr>
<tr>
<td>P-coumaric</td>
<td>7.42</td>
<td>5.53</td>
</tr>
<tr>
<td>Cinnamic</td>
<td>6.23</td>
<td>3.42</td>
</tr>
</tbody>
</table>

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Therefore, no acute oral toxicity was proven for the extracts or their mixture up to the dose of 5 g/kg b.w., and their LD₅₀ were believed to be more than 5 g/kg b.w. Hence, studies were carried out with 1/10 of the LD₅₀ as therapeutic dose (500 mg/kg) according to OECD-425 guidelines.

**Determination of biochemical parameters**

**Plasma glucose, glycosylated hemoglobin and liver glycogen levels**

Data presented in Table 3 and Figure 1 indicate that the levels of fasting plasma glucose (FPG) of untreated diabetic rats were significantly increased and amounted to 212.9% of the normal control values. Administration of different treatments partially...
reversed the increase in FPG levels reaching 146\% (MLE), 130.3\% (JLE), and 127.4\% (MLE+JLE). On the other hand, treatment with gliclazide normalized FPG levels of diabetic rats.

Similar results were obtained for HbA1c\% as shown by data compiled in Table 3 and given in Figure 1. The HbA1c\% levels of untreated diabetic rats were highly increased to reach 194.4\% of normal control, while the treatment with MLE, JLE, mixture or gliclazide ameliorated this elevation, where the HbA1c\% levels reached only 155.6, 136.1, 133.3 and 125\%, respectively.

Blood oxidative status

Table 4 and Figure 2 illustrate the effects of different treatments on plasma lipid peroxides measured as thiobarbituric acid reactive substances (TBARS), where they were highly elevated in untreated diabetic

As shown in Table 3 and Figure 1, liver glycogen of fasted untreated diabetic animals decreased significantly to reach 48.6\%. All treatments increased its levels reaching 78.7\%, 73.6\%, 74.5\% and 80.2\% for MLE, JLE, mixture and gliclazide, respectively, whereas none of them returned the levels to normal.

**Table 4. Mean level ± standard error (SE) of plasma thiobarbituric acid reactive substances (TBARS), serum nitric oxide (NOx), erythrocyte glutathione (GSH), Cu/Zn superoxide dismutase (Cu/Zn-SOD) and catalase (CAT) in differently treated groups at the end of the experimental period**

<table>
<thead>
<tr>
<th>Group</th>
<th>TBARS (nmol/L)</th>
<th>NOx (µmol/L)</th>
<th>GSH (mg/dL)</th>
<th>Cu/Zn-SOD (U/mg Hb)</th>
<th>CAT (U/mg Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N n=10</td>
<td>6.59±0.50</td>
<td>79.5±4.85</td>
<td>27.5±2.20</td>
<td>14.17±0.60</td>
<td>70.22±5.32</td>
</tr>
<tr>
<td>D n=9</td>
<td>15.45±1.03\a</td>
<td>184.3±9.69\a</td>
<td>15.0±1.26\a</td>
<td>7.88±0.68\a</td>
<td>37.7±2.70\a</td>
</tr>
<tr>
<td>D + MLE n=9</td>
<td>9.20±0.81\b</td>
<td>131.9±4.90\b</td>
<td>22.3±1.55</td>
<td>10.01±0.66\a</td>
<td>65.2±5.90\b</td>
</tr>
<tr>
<td>D + JLE n=10</td>
<td>10.90±0.73\a,b</td>
<td>116.0±8.41\a,b</td>
<td>24.1±2.37\b</td>
<td>10.42±0.54\ab</td>
<td>69.6±4.68\b</td>
</tr>
<tr>
<td>D + (MLE + JLE) n=10</td>
<td>10.81±0.39\a,b</td>
<td>98.7±7.3\b</td>
<td>25.6±1.75\b</td>
<td>14.86±0.55\b</td>
<td>73.0±3.15\b</td>
</tr>
<tr>
<td>D + GLZ n=9</td>
<td>11.08±0.73\a,b</td>
<td>135.7±11.9\a,b</td>
<td>16.9±0.99\a</td>
<td>9.27±0.30\a</td>
<td>50.60±4.1\b</td>
</tr>
</tbody>
</table>

p<0.05 (using one-way ANOVA followed by Tuckey’s HSD post-hoc test); \#significant difference from normal control group; \*significant difference from diabetic control group; n = number of animals in each group.

Figure 2. Changes of plasma thiobarbituric acid reactive substances (TBARS), serum nitric oxide (NOx), erythrocyte reduced glutathione (GSH), and the activities of Cu/Zn-superoxide dismutase (SOD) and catalase (CAT) in STZ/NA-diabetic (D) rats and in diabetic rats treated for 8 weeks with either mulberry leaf extract (D+MLE), jackfruit leaf extract (D+JLE), combined extracts [D+(MLE+JLE)] or gliclazide (D+GLZ).
rats compared to the normal control group. Administration of different extracts and gliclazide decreased TBARS levels. Unexpectedly, only MLE treatment went through normalizing TBARS levels.

Data shown in Table 4 and Figure 2 indicate the massive increase of serum NOx levels in untreated diabetic rats, which reached approximately 2.3-fold that of the normal control values. This was significantly decreased by all treatments, as they became only 1.65-, 1.45-, 1.24- and 1.7-fold for MLE, JLE, mixture and gliclazide, respectively. This also shows that only the mixture rather than any other treatment succeeded in normalizing NOx values.

Table 4 and Figure 2 show the change in blood oxidative status of diabetic treated and untreated rats, where blood GSH levels were remarkably decreased in diabetic untreated and returned to normal in JLE and mixture treated ones, but neither MLE nor gliclazide treatment was capable of returning GSH values to normal. However, MLE showed a better ameliorating effect (81.1% of normal) than gliclazide (61.5% of normal), which showed no significant difference compared with untreated diabetic rats.

As indicated in Table 4 and Figure 2, the Cu/Zn-SOD levels were markedly decreased in diabetic untreated rats. Significant elevations of Cu/Zn-SOD levels were shown by all treatments except for MLE and gliclazide, which caused no significant elevation in Cu/Zn-SOD levels than the level in the group of untreated diabetic rats, where they reached only 70.6% and 65.4% of normal control, respectively, whereas treatment with JLE showed more amelioration (73.5% of normal control). A surprising ameliorating effect of the mixture, supporting the idea of having a synergistic activity, was obvious, as it succeeded in raising Cu/Zn-SOD levels to more than that of normal controls (105%).

Table 4 and Figure 2 delineate the remarkable decrease in erythrocyte CAT levels in untreated diabetic rats compared to normal. Treatment with MLE, JLE or their mixture restored the CAT levels back to normal. Although gliclazide treatment increased CAT levels, it was nonsignificant.

**Analysis of total genomic DNA damage**

The protective effects of different treatments on the total genomic DNA damage induced by STZ-induction of diabetes in Sprague-Dawley rats after 8 weeks of treatment are shown in Table 5 and Figure 3a and 3b. As shown in Figure 3a and 3b, severe damage was observed in the DNA of the untreated diabetic rat livers (lane 2, L2), which represented the untreated diabetic control group (D). We observed DNA release and migration in the form of smear shape indicating necrosis and also in the form of bands that indicates apoptosis. These forms of released DNA (damage) disappeared in all lanes that represent the other treated groups, L3: D + MLE; L4: D + JLE; L5: D + (MLE + JLE) and L6: D + GLZ, which appeared nearly the same as the normal control (L1: normal control).

**DISCUSSION**

In the present study, treatment of diabetic rats for 8 weeks with hot water mulberry leaf extract (MLE) caused a significant decrease in FPG levels compared to diabetic control. The current results were in accordance with many previous studies (7,36-38). *Morus alba* is postulated to exert its hypoglycemic action through α-glucosidase inhibitory activity, which is attributed to the presence of D-glucose analogs 1-deoxynojirimycin and its derivatives (39). Moreover, other active constituents including piperridine alkaloid and glycoproteins from the *Morus* root bark and/or leaf extract have been used as antidiabetic agents (40), besides the presence of a variety of flavonoids and other phenolic compounds, many of which were assayed in our study, including the potentially active constituents chlorogenic acid and rutin, which might account for as much as half of the observed antidiabetic activity of *Morus alba*, as shown in a previous study (41). Our findings also complied with those of Mohammadi and Naik (3), who directly related the improvement in the number of β cells after treatment with *Morus alba* extract to its antidiabetic activity. This effect might also be attributed to rutin, which possesses stimulatory effects on the insulin secretory response of the islets of Langerhans, in addition to an ameliorative effect on the integrity of β
Table 5. **Protective effect of different treatments on total genomic DNA damage in Sprague-Dawley rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Intact DNA (4000 bp)</th>
<th>800 bp</th>
<th>600 bp</th>
<th>400 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>147</td>
<td>205</td>
<td>203</td>
<td>146</td>
</tr>
<tr>
<td>D + MLE</td>
<td>255</td>
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<tr>
<td>D + JLE</td>
<td>240</td>
<td>-</td>
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<tr>
<td>D + (MLE + JLE)</td>
<td>233</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>D + GLZ</td>
<td>254</td>
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N = normal control; D = diabetic control; D + MLE = mulberry leaves extract-treated diabetic rats; D + JLE = jackfruit leaves extract-treated diabetic rats; D + (MLE + JLE): mixture-treated diabetic rats; D + GLZ = gliclazide-treated diabetic rats; bp: base pairs

Figure 3a. **Digital photograph of DNA electrophoresis of liver tissues shows the protective effects of different treatments against STZ, where**, L1: normal control (N); L2: diabetic control (D); L3: D + MLE; L4: D + JLE; L5: D + (MLE + JLE); L6: D + GLZ and M: DNA marker.

![Digital photograph of DNA electrophoresis of liver tissues](image)

Figure 3b. **Analytical peaks for DNA lanes which resemble the same lanes in Figure 3a using Biogene software.**

![Analytical peaks for DNA lanes](image)
cells (42). The current results also agreed with those of Hamdy et al. (4), who showed that *Morus alba* exerted its hypoglycemic effect by controlling oxidative stress, increasing glycogen levels, preventing anaerobic glycolysis and improving hepatic carbohydrate metabolism, by enhancing the activities of hexokinase, glucose-6-phosphate dehydrogenase and lactate dehydrogenase.

In the current work, treatment of diabetic rats for 8 weeks with the aqueous extract of jackfruit leaves (JLE) partially reversed the elevated FPG levels toward normal control values. This result complied with those of Shahin et al. (43) and Fernando and Thabrew (44), who proved that *Artocarpus heterophyllus* aqueous extract could exert significant extra-pancreatic effects through inhibiting insulinase activity, thus inhibiting the breakdown of insulin in the circulation. Another possible mechanism through which JLE exerts its hypoglycemic action is the α-amylase inhibitory activity, which was previously reported, both *in vitro* and *in vivo*, in rat plasma (45). The α-glucosidase inhibitory activity of JLE may be attributed to the presence of considerable amounts of vitexin flavonoid, which has already been reported to possess glucosidase inhibitory activity *in vitro* (46) or *in vivo* (47).

Regarding long term control of hyperglycemia, in our experimental model of diabetes, at the end of the experimental period it was shown that glycated hemoglobin (HbA1C %) was significantly elevated in untreated diabetic group and this elevation was significantly counteracted by all treatments. These results were consistent with those of Mohammadi and Naik (3) and Omar et al. (9). This could be attributed to the reported hypoglycemic effects of both plants.

The liver glycogen level may be considered as the best marker to assess the anti-hyperglycemic activity of any drug (42). In the present study, significantly low fasting liver glycogen levels (approximately half) in the untreated diabetic rats were observed. These findings agreed with those of previous studies (42,48). The decrease in hepatic glycogen observed in this study might be due to the inactivation of glycogen synthase system, which might have happened due to the lack of insulin in type 2 diabetic state (49). In our study, after 8 weeks of treating diabetic rats with MLE and/or JLE, a marked elevation in liver glycogen levels was observed. These observations complied with those of Hamdy et al. (4) and Fernando and Thabrew (44). The latter suggested that the increased glycogen synthesis in liver and muscles of diabetic rats following administration of aqueous extract of *Artocarpus heterophyllus* leaves could have been mediated via increased concentration of insulin resulting from the inhibition of insulinase activity by the extract (44). This shows the possible way of antidiabetogenic action of these plant extracts that may be through the improvement of glycogenesis process in muscles and liver (48).

There is evidence that diabetes induces changes in the activities of antioxidant enzymes in various tissues. In DM, oxidative stress seems mainly to be due to an increased production of free radicals and/or a sharp reduction of antioxidant defense (50) and it is likely to be involved in the progression of pancreatic β cell dysfunction (51). The results of the current study clearly showed a state of oxidative stress, obvious in the untreated diabetic group, which showed significant increase in TBARS and NOx levels and significant decrease in enzymatic (SOD and CAT) and nonenzymatic (GSH) antioxidants. These findings were in accordance with numerous studies that revealed lower antioxidant and enhanced peroxidative status in type 2 DM (52,53).

Streptozotocin-induced hyperglycemia resulted in increased lipid peroxidation and protein carbonyls in red blood cells and other tissues (54). The assay of TBARS measures malondialdehyde (MDA), the end product of lipid peroxidation, present in the sample, as well as MDA generated from lipid hydroperoxides by the hydrolytic conditions of the reaction (55). In the current study, the TBARS levels of T2 diabetic rats was found to be increased at the end of the experimental period and this finding complied with the previous findings reported by Nwanjo et al. (56) in STZ diabetic rats. Nitric oxide is believed to participate in the regulation of the oxidation/reduction potential of various cells and may be involved in either the protection against or the production of oxidative stress within various tissues depending on its
concentration (57). In the present study, the concentration of serum nitric oxide was significantly increased in the untreated diabetic rat group in comparison to the normal control group. These findings are consistent with those of Yegin et al. (58). Increased NO activity could be attributed to increased nitric oxide synthase expression due to the high glucose level (59). Glutathione is the main source of red blood cell antioxidant protection and its redox status is often used as a measure of oxidative stress (60). In the present study, the erythrocyte GSH levels were found to be significantly reduced in diabetic rats compared with normal control. This result is consistent with previous studies (61,62) in STZ/NA diabetic rats. Glutathione depletion could be attributed either to its decreased synthesis due to glycation of the key synthesizing enzyme gamma-glutamylcysteine synthetase (g-GCS), or increased degradation of GSH under diabetic conditions. Moreover, hyperglycemia results in increased enzymatic conversion of glucose to the polyalcohol sorbitol with concomitant decreases in NADPH and glutathione (60). Antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), constitute an important protective mechanism against the ROS and their effectiveness varies with the stage of development and other physiological aspects of the body. The current study showed a significant decrease in both SOD and CAT activities in erythrocytes of untreated diabetic rats compared to normal control. These data are in agreement with previous studies (64,65). A possible explanation for the current results is reduced antioxidant protection in type 2 DM and/or greatly increased amounts of free radicals that overwhelm the defense system (66). Another possible explanation is that the decreased activity of Cu-Zn superoxide dismutase is related to either increased free radical production causing oxidation followed by denaturing of the enzyme, or alternatively glycation of the enzyme with resulting inhibition of enzymatic activity (67). Watanab (68) attributed the reduction of CAT activity to the higher levels of H2O2 produced by polymorphonuclear leukocytes in type 2 DM. Yan et al. (69) investigated the inactivation of SOD and CAT by sugars and the loss of antigenicity that was monitored by the loss of activity.

The present study showed that treatment of diabetic rats with JLE, as well as the mixture significantly improved SOD activity, reaching 73.5% and 105% of normal control, respectively. Although MLE caused an increase in SOD activity that reached 70.6% of normal control, this increase was not significant. The activity of catalase enzyme was also significantly improved by different treatments reaching 93%, 99% and 104% of normal control for MLE, JLE and the mixture, respectively. Enhancement of antioxidant enzymatic activities in vivo reflects the antioxidant potency of both plants.

Besides, the effects of these treatments on oxidation and lipid peroxidation biomarkers (GSH, SOD, CAT, TBARS and NOx) were found to be better than those of gliclazide. The results of ameliorating oxidative stress in Morus alba treated diabetic rats were in accordance with those of previous studies (4,70). The antioxidant effect of MLE might be attributed to the presence of many flavonoids and phenolic compounds, identified in MLE in this study, including e-vanillic acid, 3-OH-tyrosol, benzoic acid, hisperedin, and rutin. Polyphenolic compounds were shown to have radicals scavenging activity and an ability to activate key antioxidant enzymes in vivo and thus breaking the vicious cycle of oxidative stress and tissue damage (71). The ameliorative effect of mulberry-treatment on the oxidative state in vivo was also previously demonstrated (37,70). The in vitro antioxidative properties were proven by Katsube et al. (72) through LDL oxidation, 1,1-diphenyl-2-picrylhydrazyl (DPPH)3 radical scavenging, and Folin-Ciocalteu assays. Concerning JLE, the presence of flavonoids and other phenolic compounds, including vitexin, hisperetin, quercetin, chionic acid, e-vanillic acid and protochateuc acid, identified in this study, might have played an important role in the observed antioxidant effects. Vitexin has been reported to have a potent free radical scavenging activity (73). It was proven that 40 mg/kg dose of vitexin had the same antioxidant capacity as vitamin E (74), whereas quercetin exerted antioxidant effects in STZ-induced experimental diabetes (75). Moreover, cyclo-heterophyllin and artonins A and B, prenylflavones previously isolated from Artocarpus heterophyllus
Lam., serve as powerful antioxidants against lipid peroxidation when biomembranes are exposed to oxygen radicals (76).

In the current study, gliclazide also was shown to possess some antioxidant properties. However, its ameliorative effect on enzymatic SOD and CAT, and nonenzymatic antioxidant GSH, was not significant. On the other hand, it significantly decreased the elevation in TBARS and NOx caused in diabetic rats, but it failed to normalize it totally. These results are in accordance with many studies that proved the antioxidant properties of gliclazide (77,78).

The liver plays a unique role in controlling carbohydrate metabolism by maintaining glucose concentrations in a normal range over both short and long periods of time (79). In 1980, Laguens et al. (80) reported the hepatotoxic effect of STZ independently of its diabetogenic action. In the current study, DNA analysis by gel electrophoresis showed severe DNA damage to the liver tissues of diabetic rats; this result is in accordance with that of Kim et al. (81). Liver DNA damage observed in our study might have resulted from the hepatotoxic effect of STZ. This result was supported by the previous studies performed by Schmezer et al. (82), who observed that STZ had induced DNA strand breaks and mutations in the liver and kidney of mice. Another explanation for the observed DNA damage is the state of oxidative stress which is also known to cause massive DNA fragmentation. Moreover, oxidative damage to hepatic nuclear DNA increases in diabetic state and this increase was correlated with glycation stress (83).

In this study, treatment of STZ/NA diabetic rats for 8 weeks either with MLE, JLE or their mixture significantly reduced the liver DNA damage. Since the DNA damage is most probably due to STZ-induced oxidative stress, or the oxidative stress happened as a consequence of diabetic state, it suggests that this protective effect against DNA damage was most probably due to the antioxidant properties previously proven for both plants. Gliclazide treatment also significantly reduced DNA fragmentation in livers of diabetic rats and this result was in agreement with that of Kim et al. (81) in STZ-diabetic mice. The reduction in DNA fragmentation might be attributed to the free radical scavenging activity reported for gliclazide (78).

Finally, the probable synergistic effect motivated the use of a mixture of both plants in the treatment of diabetic rats in this study. However, the synergistic effect of the mixture was highly pronounced only in case of elevating the diminished SOD levels and in lowering the elevated NOx levels in diabetic rats, while other parameters were very similar to or slightly better than with JLE. In our study, we observed that JLE was always more effective than MLE, except for two cases, namely, TBARS and liver glycogen, in which MLE was more effective.

**CONCLUSION**

In conclusion, the current work demonstrated that treatment with hot water extracts of mulberry leaves, jackfruit leaves or mixture of both is beneficial in treating diabetes mellitus, where they lowered hyperglycemia and oxidative stress, which, in turn, may have a consequent role in preventing the progression of diabetic complications. The bioactive component(s) responsible for the observed activities are not precisely known but it may be one or more of the phytochemical constituents established to be present in the leaf extracts. The presence of flavonoids and other phenolic compounds in both extracts suggests that they might be the constituents responsible for these activities. Therefore, the present study confirmed the results of earlier studies that showed the importance of using both these plants to ameliorate diabetes and its complications. To the best of our knowledge, this is the first study that compared the effect of these two plants to each other and to a mixture of both, on a type 2 diabetic animal model. However, this study did not precisely define the certain active constituent(s) that is/are responsible for the hypoglycemic activities of both plants. Neither did it discover new mechanism(s) of action which may underlie these activities. Further mechanistic studies are needed to identify the specific active constituents responsible for the biologic activities observed for
both plants. Clinical studies are also needed to prove the efficacy and safety of these medicinal plants in humans.

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REFERENCES


SUMMARY

Elevated LDL-cholesterol level is a major risk factor for cardiovascular disease (CVD) and atherosclerosis. LDL-cholesterol is cleared from circulation by the LDL receptor (LDL-R). Proprotein convertase subtilisin kexin 9 (PCSK9), an enzyme secreted into the plasma by the liver, binds the LDL-R in complex which is then subject to endocytosis and degradation in lysosomes. PCSK9 inhibitors prevent LDL-R degradation, the LDL-R recycling is preserved, with a consequent increase in receptor density on the hepatocyte surface and LDL-cholesterol clearance. In recent years, human monoclonal antibodies against PCSK9 have been identified as an innovative lipid-lowering strategy. Clinical trials confirmed that treatment with PCSK9 antibodies combined with statins, as well as in monotherapy produces profound reductions in total, LDL-cholesterol and lipoprotein(a), with a similar level of safety and survival benefit compared with no anti-PCSK9 treatment. Confirmation of these findings in ongoing, long-term trials will establish the role of these novel agents in reducing the risk of CVD.

INTRODUCTION

Hypercholesterolemia is the most important risk factor for atherosclerosis and cardiovascular disease (CVD) (1). Inherited autosomal dominant hypercholesterolemia (ADH) is associated with markedly elevated levels of cholesterol and occurs at a frequency of 1/500 worldwide, and it is linked to heterozygous dominant mutations in the genes encoding the low density lipoprotein receptor (LDL-R) with over 1600 mutations identified (2). Statins have been the first-line drugs for lowering cholesterol since the late 1980s. Statins inhibit the rate-limiting step of cholesterol synthesis catalyzed by hydroxymethylglutaryl coenzyme A reductase (HMGCoA reductase) reducing the incidence of atherosclerosis. They upregulate the transcription factor SREBP-2, which then stimulates the expression of LDL-R resulting in increased LDL-cholesterol uptake by hepatocytes, and consequently lowering
circulating levels of LDL-cholesterol (3). Although statins reduce cardiovascular events by 25%-40%, they often lead to suboptimal levels of LDL-cholesterol, especially in patients with ADH, and are not well tolerated due to unwanted side effects (4, 5). Other drugs are also being considered to manage hyperlipidemia such as fibrates, ezetimibe, colestevam, torcetrapib, avasimibe, implitapid, and niacin. Fibrates decrease fatty acid and triglyceride levels by stimulating the peroxisomal β-oxidation pathway, ezetimibe selectively inhibits intestinal cholesterol absorption, cholestyramine, colestipol and colestevam sequester bile acids, torcetrapib inhibits cholesterol ester transfer protein, avasimibe inhibits acyl-CoA:cholesterol acyltransferase, implitapid inhibits microsomal triglyceride transfer protein, and niacin modifies lipoproteins (6).

Many bioactive secretory proteins, including polypeptide hormones and enzymes, are initially produced as inactive precursors, and conversion of an inactive secretory precursor into active product(s) is catalyzed by a special group of proteases denoted as proprotein convertases (7). Proprotein convertase subtilisin kexin 9 (PCSK9) was first reported in early 2003 (8). It is highly expressed in liver hepatocytes and found to represent the third familial hypercholesterolemia gene, with the LDL-R and apolipoprotein B (ApoB) genes being the other two (9, 10). After the discovery of PCSK9, its relationship to CVD has been well documented (11, 12). Moreover, heterozygote African nonsense loss-of-function mutation of PCSK9 correlates with a remarkable 88% reduction in CVD risk when compared with noncarriers (13). In addition, because mammals can survive and stay healthy without PCSK9, it was found that 2 complete loss-of-function mutations caused an amazingly low level of circulating LDL-cholesterol of about 0.4 mmol/L (14, 15). Since PCSK9 binds the hepatocyte-derived LDL-R, disrupting this protein-protein interaction prevents LDL-R degradation, and raising the LDL-R levels lowers LDL-cholesterol levels and protects from the development of atherosclerosis (16, 17).

Figure 1. LDL-cholesterol metabolism in the liver without PCSK9 binding to the LDL receptor: LDL receptor is recycled and more LDL-cholesterol can be removed from the blood.
BIOLOGY, EXTRACELLULAR AND INTRACELLULAR ACTIVITIES OF PCSK9 IN ANIMAL MODELS

The human 22-kb gene PCSK9 is located on the small arm of chromosome 1p32 and contains 12 exons and 11 introns and encodes a 692–amino acid (aa) proteinase K-like serine protease named PCSK9 (10, 18). In the adult, PCSK9 is highly expressed in liver hepatocytes and less in the small intestine and kidney, and has no other substrate than itself, and its activity is related to its binding to specific target proteins and to escort the resulting complex toward intracellular degradation compartments (19). The first PCSK9 target to be identified is the LDL-R at the surface of liver hepatocytes (20, 21). After binding to the LDL-R, the PCSK9-LDL-R complex inhibits normal process that has resulted in the acidic pH of endosomes and causes allosteric dissociation of the LDL-R and its recycling to the cell surface, as well as direction of the LDL-cholesterol to the lysosomes for degradation (Figure 1) (22). In contrast, the complex PCSK9-LDL-R does not dissociate at acidic pH but is rather more tightly associated and then moved to lysosomes for degradation (Figure 2) (23).

Figure 2. LDL-cholesterol metabolism in the liver with PCSK9 binding to the LDL receptor: LDL receptor is degraded and less LDL-cholesterol can be removed from the blood.
degradation (32). Recently, the critical requirement for the M2 domain of the CHRD in the extracellular pathway has been described, but not for the intracellular one (33). However, both the intracellular and the extracellular LDL-R degradation activities of PCSK9 require the presence of the CHRD because the lack of this domain does not traffic to lysosomes and is likely recycled to the cell surface (33, 34). In addition, Annexin A2 (AnxA2), a functional inhibitor of PCSK9 that targets the CHRD, also acts as an endogenous regulator of LDL-R degradation in extrahepatic tissues (35). No specific inhibitor of the intracellular function of PCSK9 has yet been identified because studies suggest that the two intracellular/extracellular pathways show different dynamic subcellular co-localization patterns of PCSK9 and LDL-R (36).

Transgenic mice overexpressing PCSK9 under an Apoe promoter are viable, fertile and severely hypercholesterolemic, and develop accelerated atherosclerosis with larger plaque size when fed a regular chow diet (21, 26). The circulating LDL-cholesterol in these mice is only 5-fold increased, although the LDL-R is not detected in the liver, compared with 15-fold increase in LDLR-knockout mice, suggesting that the LDL-R are expressed in extrahepatic tissues and those LDL-R are not regulated by circulating PCSK9 (7, 26). Pig model of a human PCSK9 mutant also had markedly reduced levels of hepatic LDL-R and severe hypercholesterolemia, and these minipigs developed spontaneous progressive atherosclerotic lesions (37). In contrast, complete PCSK9-knockout mice are viable and fertile exhibiting severe hypocholesterolemia with a 40%-80% drop in total cholesterol and LDL-cholesterol compared to liver-specific PCSK9-knockout mice that exhibit about 27% less circulating cholesterol levels, indicating that liver PCSK9 contributes to about 70% of the cholesterol phenotype (26, 27, 38). Moreover, PCSK9-knockout mice have reduced plasma levels of sphingomyelin and ceramides, known risk factors for CAD (39). Since PCSK9 may target other receptors, in PCSK9-knockout mice VLDL-R is also upregulated and associated with a 2-fold decrease in postprandial triglyceride levels, suggesting a role for PCSK9 in triglyceride metabolism, possibly via its ability to enhance the degradation of VLDL-R (40). Circulating PCSK9 also regulates the levels of its cell surface receptors in adipose tissue and lack of PCSK9 increases the surface expression of VLDL-R that facilitates triglyceride hydrolysis and free fatty acid uptake in visceral adipocytes (41). VLDL-R is upregulated in perigonadal depots of PCSK9-knockout mice, and perigonadal fat is part of the visceral adipose tissue, which correlates directly with metabolic disease and CAD.

In Anxa2-knockout mice (Annexin A2 is a natural extrahepatic inhibitor of PCSK9), plasma PCSK9 doubles and LDL-R decreases by about 50% in extrahepatic tissues such as adrenals, small intestine and colon (35, 42). PCSK9 also regulates the LDL-R protein levels in intestinal cell lines and has a role in the transintestinal reverse cholesterol excretion from the small intestine (43, 44). PCSK9 is highly expressed in pancreatic β-cell lines and PCSK9-knockout mice express more LDL-R in pancreatic islet cells, are glucose-intolerant and may be at risk to develop diabetes mellitus (8, 45).

**PCSK9 INHIBITORS – CLINICAL TRIALS**

The animal model of the loss-of-function mutations in PCSK9 that resulted in markedly reduced LDL-cholesterol plasma levels facilitates investigation of a new class of lipid-lowering therapies that might prevent CAD (46). Each LDL-R normally recycles approximately 150 times and monoclonal antibody binding and inhibition of PCSK9 prevents lysosomal degradation of the LDL-R. The LDL-R recycling is preserved, with a consequent increase in receptor density on the hepatocyte surface and LDL-cholesterol clearance. International guidelines have promoted increasingly lower treatment goals for LDL-cholesterol to reduce cardiovascular risk (1, 47). Although statins have acceptable efficacy and safety profiles, more than one half of cardiovascular events are not prevented by these drugs, and up to 40% of patients receiving statins are not able to reach target LDL-cholesterol levels with current guideline recommendations (48). While some of those patients
were treated with submaximal doses, had side effects and pharmacological interactions, a limited additional 6% decrease in LDL-cholesterol was observed with doubling of the statin dose (49). In recent years, human monoclonal antibodies against PCSK9 have been identified as an innovative lipid-lowering strategy. Many monoclonal antibodies including evolocumab, alirocumab and bococizumab have been developed within the past 5 years. These PCSK9 inhibitors, administered subcutaneously in monthly or semimonthly injections, combined with statins, as well as in monotherapy, provided benefits in reducing atherogenic lipid fractions in several phase 3 studies (50-52). Results of a meta-analysis of 24 randomized trials that evaluated the effects of PCSK9 antibodies in 10 159 adults with hypercholesterolemia, previously treated with statins who had not met target LDL-cholesterol goals or who did not tolerate statins, showed that PCSK9 inhibition, compared with placebo or ezetimibe control groups, led to a 47% reduction in LDL-cholesterol (50). Compared to ezetimibe, LDL-cholesterol reduction observed with alirocumab and evolocumab was distinctly greater with an average additional 36% reduction in LDL-cholesterol from baseline. Moreover, the reduction in lipoprotein(a), another lipid fraction that contributes to atherosclerotic plaque formation, was 25% on average compared with placebo or ezetimibe. The results also showed that therapy with PCSK9 antibodies was associated with lower odds of all-cause mortality and myocardial infarction but nonsignificant reduction in cardiovascular mortality. Results were robust in sensitivity analyses that were stratified by intensity of background statin therapy or by comparator (placebo or ezetimibe). However, these results warrant cautious interpretation because the included trials were of small or moderate size with short follow up periods and small total number of events. There was also a statistically significant 30% reduction in the odds of increased creatine kinase levels with the use of PCSK9 antibodies compared with no anti-PCSK9 treatment and the rates of serious adverse events did not significantly differ with versus without anti-PCSK9 treatment, confirming the overall comparative safety of the drug. In contrast to this meta-analysis where reduction in cardiovascular mortality was not statistically significant, OSLER trials 1 and 2 showed a statistically significant reduction in a combined cardiovascular end point with evolocumab (53). In addition, the ODYSSEY LONG TERM study, which includes the largest patient population studied over 78 weeks, demonstrated a reduction in myocardial infarction with PCSK9 antibody therapy (54). However, PCSK9 antibodies have been tested predominantly in adults without the acute coronary syndrome who have hypercholesterolemia. Since statins (and ezetimibe) may induce upregulation of PCSK9 attenuating their LDL-cholesterol lowering effect, PCSK9 inhibitors may be particularly helpful in patients who do not achieve the expected LDL-cholesterol level with maximally tolerated statins or in those with statin intolerance and with very high baseline LDL-cholesterol levels (patients with familial hypercholesterolemia) (55, 56). In support to these data, the use of PCSK9 antibodies combined with statins provided benefits in terms of reducing atherogenic lipid fractions in patients with hyperlipidemia (LAPLACE-2) or heterozygous familial hypercholesterolemia (RUTHERFORD-2) (57, 58). Moreover, in GAUSS and GAUSS-2 trials, which included predominantly statin-intolerant patients, treatment with evolocumab resulted in up to 50% reduction in the LDL-cholesterol level (59). Fourteen trials comprising 4378 patients were included in the analysis of HDL-cholesterol and showed 6.3% increase in HDL-cholesterol with the use of PCSK9 antibodies compared to placebo or ezetimibe (50). Ten studies comprising 5357 patients were included in the analysis of total cholesterol and showed a 39% reduction with the use of PCSK9 antibodies compared to placebo and 24% reduction compared to ezetimibe. Recently, the US Food and Drug Administration (FDA) granted approval to PCSK9 inhibitor alirocumab for heterozygous familial hypercholesterolemia and for patients with clinical atherosclerotic cardiovascular disease, while the European Medicines Agency (EMA) has recommended approval of the PCSK9 inhibitor.
alirocumab for individuals who cannot lower their high LDL-cholesterol levels with statins or who cannot tolerate statins.

Four large cardiovascular outcomes trials exploring the ability of PCSK9 antibodies to affect clinical outcomes are ongoing. Since PCSK9 antibodies have been tested predominantly in adults without the acute coronary syndrome, the FOURIER study is assessing whether treatment with evolocumab compared with placebo reduces recurrent cardiovascular events in 27,500 adults with established CAD (60). The ODYSSEY outcomes trial will investigate the effect of alirocumab on major adverse cardiovascular events in about 18,000 patients with heterozygous familial hypercholesterolemia, established coronary heart disease, or a coronary heart disease risk equivalent, receiving treatment with statins at the maximum tolerated dose (61). In post hoc analysis of 2341 patients after 78-week follow up alirocumab reduced the level of LDL-cholesterol by 61% compared to placebo and there was evidence for reduction in the rate of cardiovascular events with alirocumab (62). Two cardiovascular outcome studies with bococizumab investigating the change in LDL-cholesterol, in some subjects to the levels well below the current guideline recommended targets, in more than 22,000 patients, are in the phase 3 program (50).

CONCLUSION

Since the discovery of PCSK9 in 2003 and its implication in LDL-cholesterol regulation, investigations have shown that PCSK9 is a key regulator of LDL-cholesterol metabolism that promotes hepatic degradation of LDL-R and reduces LDL-cholesterol clearance. The initial clinical trial experience with PCSK9 inhibitors has confirmed that treatment with PCSK9 antibodies produces profound reductions in total, LDL-cholesterol and lipoprotein(a), with a similar level of safety and survival benefit compared with no anti-PCSK9 treatment. However, PCSK9 inhibitors are monoclonal antibodies that are high molecular mass proteins complex to manufacture and potentially immunogenic, are high cost and unsuited for oral delivery. Confirmation of these findings in ongoing, long-term, pivotal trials with prespecified cardiovascular end points should provide additional data on the safety of this innovative therapy and will help establish the role of these novel agents in reducing the incidence of cardiovascular disease.
REFERENCES


SUMMARY

New insulin glargine 300 units/mL (Gla-300) is a basal insulin that provides the same number of units as Gla-100 in a third of the volume. Compared with Gla-100, Gla-300 has shown more constant and prolonged pharmacokinetic (PK)/pharmacodynamic (PD) profiles. Gla-300 has been shown to achieve similar glycemic control with less nocturnal hypoglycemia compared with Gla-100, and lower hypoglycemia at any time of day. The EDITION clinical program also provides evidence for less weight gain with Gla-300 than with Gla-100. The PK/PD profiles of Gla-300 may allow more flexibility in the timing of doses. Based on these findings, Gla-300 could be optimal treatment option for a range of people with type 1 and type 2 diabetes mellitus.

INTRODUCTION

Based on the current knowledge about the treatment of type 2 diabetes mellitus (T2DM), it is recommended to initiate insulin therapy using a basal insulin. T2DM is a pathophysiological progressive disease requiring intensification of therapy over time. Consequently, some patients will eventually need intensified insulin therapy, consisting of an intermediate- or long-acting insulin at bedtime for fasting glucose control and a short-acting mealtime insulin (administered before breakfast, lunch and dinner) for the control of postprandial hyperglycemia. This so-called basal-bolus insulin regimen is the standard of care for all people with type 1 diabetes mellitus (T1DM), with long-acting insulin analogues being used much more commonly than human intermediate-acting insulins, due to their more even profile of action leading to a reduced risk of hypoglycemia.

After the failure of an oral antihyperglycemic drug (OAD) combination therapy, basal insulin treatment is recommended to reduce the level of fasting hyperglycemia, which occurs in people with T2DM as a consequence of increased hepatic glucose production when the quantity of endogenous insulin is no longer sufficient to suppress gluconeogenesis adequately.
Hypoglycemia is the most frequent adverse reaction of insulin therapy. Insulin therapy aimed at reducing glycemic values is associated with an increased risk of hypoglycemia. The fear from hypoglycemia remains a major obstacle to achieve adequate glycemic control in both insulin-experienced and insulin-naïve patients with type 1 and type 2 diabetes mellitus.

The even and peakless profile of long-acting insulin analogues mimics the physiological basal pancreatic insulin secretion, thereby reducing the risk of hypoglycemia compared to long-acting human insulin (neutral protamine Hagedorn, NPH), with a satisfactory HbA1c-lowering effect.

The ideal basal insulin should be administered once daily and ensure a slower, more even insulin release, with little glycemic variability, an evenly distributed 24-hour effect and a low risk of hypoglycemia.

PHARMACOKINETICS AND PHARMACODYNAMICS OF INSULIN GLARGINE 300 UNITS/mL

Insulin glargine is a human insulin analogue with low solubility at neutral pH. Insulin glargine is completely soluble at pH 4. Following injection into subcutaneous tissue, the acidic solution is neutralized, leading to the formation of a precipitate from which small amounts of insulin glargine are continuously released (2).

Insulin glargine 100 units/mL (Lantus®) is indicated for the treatment of diabetes mellitus in patients who need insulin to improve their glycemic control.

Insulin glargine 300 units/mL (Toujeo®) has the same molecular composition as insulin glargine 100 units/mL but shows a more even and prolonged pharmacokinetic profile, enabling an evenly distributed daytime glucose-lowering effect with high reproducibility of action, i.e. less variability. These advantages result from structural enhancement of the subcutaneous microprecipitate (Figure 1). The microprecipitate surface area has been reduced and its compactness increased, thus facilitating a prolonged release of insulin glargine from insulin glargine 300 units/mL precipitate compared to insulin glargine 100 units/mL (3–6).

As observed in euglycemic clamp studies in patients with T1DM (a crossover study in 18 patients), the glucose-lowering effect of insulin glargine 300 units/mL after a subcutaneous injection was more stable and prolonged compared to insulin glargine 100 units/mL. At clinically relevant doses, the effect of insulin glargine 300 units/mL was sustained beyond 24 hours (up to 36 hours) (7).

In view of the above, insulin glargine 300 units/mL is not bioequivalent to insulin glargine 100 units/mL and the two are not interchangeable (Figure 2).

In a continuous glucose monitoring study conducted in 59 patients with T1DM, the mean glucose profile was consistent in patients treated with insulin glargine 300 units/mL compared to those treated with insulin glargine 100 units/mL, regardless of whether the doses were administered in the morning or in the evening (6).

After a subcutaneous injection of insulin glargine in healthy subjects and diabetic patients, insulin serum concentrations indicated a slower and significantly more prolonged absorption, without peak values, compared to human NPH insulin. The concentrations were consistent with the time profile of the pharmacodynamic activity of insulin glargine. The therapeutic steady-state is reached after 3 to 4 days of daily administration of insulin glargine 300 units/mL. After subcutaneous administration of insulin glargine 300 units/mL, the intra-subject variability, defined as the coefficient of variation for 24-hour insulin exposure, was low at steady-state (17.4%). In human subcutaneous tissue, insulin glargine is partially metabolized at the carboxyl termini of the beta-chain to form active metabolites, M1 (21A-Gly-insulin) and M2 (21A-Gly-des-30B-Thr-insulin). Pharmacokinetic and pharmacodynamic findings indicate that the effect of a subcutaneous insulin glargine injection is principally based on exposure to M1. In the majority of subjects, insulin glargine and M2 were undetectable, and when detected, their concentration was independent of the dose or the insulin glargine formulation administered. The elimination half-life of insulin glargine 300 units/mL after subcutaneous
Figure 1. The more sustained release of insulin glargine from the Gla-300 (insulin glargine 300 units/mL) precipitate compared to Gla-100 (insulin glargine 100 units/mL) is attributable to the reduction in injection volume by two thirds, which results in a smaller precipitate surface area.

Figure 2. Insulin concentration (INS), glucose infusion rate (GIR) and blood glucose (BG) after multiple doses of Gla-100 (insulin glargine 100 units/mL) and Gla-300 (insulin glargine 300 units/mL).
administration is determined by the rate of absorption from the subcutaneous tissue. The half-life is 18-19 hours, regardless of the dose (5).

CLINICAL EFFICACY

The efficacy and safety of the new insulin glargine 300 units/mL was evaluated in a comprehensive EDITION clinical program, which encompassed a wide range of T2DM patients (n=2474), including insulin-naïve patients treated with OADs (EDITION3), patients previously treated with basal insulin in combination with OADs (EDITION2) and patients on intensified insulin therapy consisting of a basal insulin and a short-acting mealtime insulin (EDITION1) (8-10). In such a large number of T2DM patients participating in the EDITION program, insulin glargine 300 units/mL proved to be just as effective in improving glycemic control over 6 months as insulin glargine 100 U/mL.

However, in the EDITION clinical program, insulin glargine 300 units/mL demonstrated superiority over insulin glargine 100 units/mL with respect to the number of confirmed (≤3.9 mmol/L) and/or severe hypoglycemic events (relative difference: 14%) at any time of day (over 24 hours) (Figure 3).

An even greater relative difference (31%) in the number of confirmed (≤3.9 mmol/L) and/or severe hypoglycemic events favoring insulin glargine 300 units/mL compared to insulin glargine 100 units/mL was observed during night time (00:00-06:00 AM) (Figure 3).

In general, these effects on the risk of hypoglycemia have been consistently observed in the clinical program throughout the treatment period, i.e. both in the titration and the dose maintenance periods (11).

DOSING

Insulin glargine 300 units/mL is administered once daily at any time, but always at the same time of day. The dosing regimen of insulin glargine 300 units/mL (dose and timing) should be adjusted individually. The recommended starting dose in T2DM patients is 0.2 units/kg daily, followed by individual dose adjustments. In the elderly (≥65 years), progressive deterioration of renal function may result in gradual and steady decreases in insulin requirements. Insulin requirements may also be lower in patients with renal and hepatic impairment. When switching from an intermediate- or long-acting insulin to insulin glargine 300 units/mL, the basal insulin dose may have to be modified and concomitant anti-hyperglycemic drugs adjusted (2).

Switching from once-daily basal insulins, including insulin glargine 100 units/mL, to once-daily insulin glargine 300 units/mL can be done unit-to-unit based on the previous basal insulin dose. When switching from twice-daily basal insulins to once-daily insulin glargine 300 units/mL, the recommended initial dose of insulin glargine 300 units/mL is 70%-80% of the total daily dose of the basal insulin that is being discontinued (2).
SAFETY

Insulin glargine 300 units/mL is not the insulin of choice for the treatment of diabetic ketoacidosis. In such cases, intravenous administration of regular insulin is recommended. In case of inadequate glycemic control or a predisposition to hyper- or hypoglycemia, prior to considering dose modification the patient’s adherence to the prescribed dosing regimen, injection sites and the proper injection technique, as well as all other relevant factors should be evaluated. Given that insulin glargine 100 units/mL and insulin glargine 300 units/mL are neither bioequivalent nor interchangeable, switching from one formulation to the other may require a dose modification and should be performed under close medical supervision (2).

There are no data on pregnancies exposed to insulin glargine from controlled clinical trials.

Insulin glargine has also been investigated in a comprehensive cardiovascular outcome study entitled ORIGIN (Outcome Reduction with Initial Glargine INtervention). This multicentre, randomized clinical study included T2DM patients (N=12,537) at a high cardiovascular risk (12). Patients were randomized (1:1) to either insulin glargine or the standard of care. The first co-primary efficacy outcome was the time to the first occurrence of cardiovascular death, nonfatal myocardial infarction or nonfatal stroke, whereas the second co-primary efficacy outcome was the time to first occurrence of any of the first co-primary events, or a revascularization procedure, or hospitalization for heart failure. Insulin glargine did not alter the relative risk of cardiovascular disease and cardiovascular mortality when compared to the standard of care.

CONCLUSION

Based on the clinical and pharmacological indicators, insulin glargine 300 units/mL is an advancement in the treatment of T1DM and T2DM in patients who require either initiation or transition of insulin therapy. In both T1DM and T2DM, it is important to meet basal insulin requirements using a peakless long-acting insulin analogue, which minimizes the risk of hyperglycemia and hypoglycemia (8-10, 13, 14). It is precisely hypoglycemia that is an important obstacle to achieve diabetes control goals, and insulin glargine 300 units/mL, being a long-acting basal insulin of low variability and prolonged even activity, facilitates glycemic control with a low risk of hypoglycemia. In a broad population of T2DM patients, insulin glargine 300 units/mL provides comparable glycemic control to that provided by glargine-100, with less hypoglycemia at any time of day (24 h), and a more pronounced reduction in hypoglycemia during the night, and importantly during the titration period (first 8 weeks) (11).

The flexibility in dosing time allows for insulin glargine 300 units/mL to be administered up to 3 hours before or after the usual dosing time, thus ensuring further improvement in the patient quality of life while maintaining a good glycemic control.

In addition, in patients treated with insulin glargine 300 units/mL, the mean observed weight change at the end of a 6-month period in the clinical program was less than 1 kg. Given that insulin therapy is usually associated with weight gain, this result, indicating an almost weightneutral effect of insulin glargine 300 units/mL, is an important additional treatment benefit, especially in patients with T2DM.
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