SUPPLEMENTARY MATERIAL

Evaluation of *Morus nigra* L. and *Bauhinia variegata* L. Leaves as Egyptian Folk Medicine Remedies used in Treatment of Diabetes

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Abstract

Black mulberry (*Morus nigra* L.) and *Bauhinia variegata* L. are used in the Egyptian folk medicine for their hypoglycemic effects. The standardized ethanolic extracts of both plants caused a significant decrease in fasting blood glucose level at two different doses (250 and 500 mg/kg) in streptozotocin-induced diabetic rats' model. Further, *in vitro* antioxidant activity and α-glucosidase inhibition assays were conducted as well as measurement of insulin levels and biomarkers for both liver and kidney functions in the treated animals. Beneficiary effects of BMLE and BVLE in the treatment of diabetes were found not to be limited to hypoglycemic effect but include preventing liver and kidney tissue damage that is associated with diabetes. A strong inhibition of the α-glucosidase enzyme by both extracts may be a contributing mechanism in the overall anti-diabetic effect that was observed. Further detailed study is needed in the future to explore the mechanism of action of both plants.

Keywords: *Morus nigra*; *Bauhinia variegata*; Diabetes; Insulin; Liver Functions; Kidney Functions.
1. Experimental

1.1. General and Chemicals

Streptozotocin, gliclazide, and 1-deoxyojirimycin were purchased from Sigma Co., USA. α-glucosidase enzyme and 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, acarbose, 4-nitrophenyl α-D-glucopyranoside (pNPG) were purchased from Sigma-Aldrich, Germany. Biodiagnostic transaminase kits (AST and ALT), creatinine, urea and insulin kits (Bio-merieux Co., France) were used to determine serum levels of different biochemical markers in the treated animals. Solvents used for HPLC analysis were of HPLC grade and were purchased from Sigma-Aldrich. Gallic acid and quercetin were obtained from E-Merck (Darmstadt, Germany). External reference standards for identification of major flavonoids and phenolic acids (rutin, quercetin, quercetin, hesperidin, chlorogenic acid, ρ-coumaric acid, cinnamic acid) were kindly supplied by Agricultural Research Center, Giza, Egypt.

1.2. Plant materials

Leaves of Morus nigra L. were collected in April 2013 from a private garden along Alexandria – Cairo Desert Road, Egypt, while leaves of B. variegata Linn. were collected from Orman Botanical garden, Cairo, Egypt. Both plants were kindly authenticated by Dr. Mohammed El-Gebaly, Department of Botany, National Research Center. A voucher specimen (1442016-I) for Morus nigra and (1442016-II) for B. variegata were deposited in the Herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

1.3. Preparation of plant extracts

The air-dried powdered leaves of black mulberry (1kg) were extracted with 70% ethanol by cold maceration till exhaustion (2 L X 10). The collected extract was filtered and evaporated to dryness using rotary evaporator at 40 °C to yield 160 g of the dark green residue of BMLE.

Leaves of B. variegata (900 g) were similarly extracted, concentrated to yield 60 g of the reddish brown residue of BVLE.
1.4. Determination of total phenolics and flavonoids content

The total phenolic content of both leaves extracts was measured using the Folin-Ciocalteu colorimetric method modified by Wolfe et al (2003) and was expressed as milligrams of gallic acid equivalents per gram of extract (mg of GAE/g) using a calibration curve plotted using serial dilutions of gallic acid.

The total flavonoids content was determined using AlCl₃ method (Ivanova et al. 2010) and was expressed as milligrams of quercetin equivalents per gram of extract (mg of QE/g) using a calibration curve plotted using serial dilutions of quercetin. Spectrophotometric assays for total phenolics and flavonoids were done in triplicate. All assays were measured using 96-wells microplate reader (Sunrise Tecanreader, Germany).

1.5. HPLC analysis of flavonoids and phenolic acids

Hewlett-Packard Series 1100 HPLC system (Waldbronn, Germany) equipped with diode array detector and an analytical column (Lichrosorb C₁₈ column, 250 mm x 4 mm, 5 µm, Merck, Darmstadt) was used for identification of flavonoids at λ=330 nm and phenolic acids at λ=280 nm.

The analysis of the flavonoids was done using a mobile phase consisting of 50 mM H₃PO₄, pH 2.5 (solution A) and acetonitrile (solution B) as follows: isocratic elution 95% A/5% B, for 5 min followed by linear gradient from to 50% A/50% B over 20 min and then composition of mobile phase was held constant for 5 min. Eluted compounds were monitored at 330 nm.

Analysis of phenolic acids was achieved with a solvent system consisting of A (aqueous acetic acid 2.5%), B (acetonitrile) with a multistep gradient starting at 95% A / 5% B to reach 90% A / 10%B at 5 min then 70% A / 30% B at 10 min; 50% A / 50% B at 13 min to finally reach 100% B at 25 min. The solvent flow rate was 1 mL/min. The injection volumes were 5 µl. Elution was monitored at 280 nm. Flavonoids and phenolic acids were identified by comparing their retention times to that of a set of external standards analyzed under the same conditions.

1.6. Determination of 1-DNJ content in BMLE

UPLC/ESI-MS/MS assay was used to determine 1-DNJ content in BMLE extract. Acquity UPLC BEH shield equipped with RP C₁₈ column (150 mm x 2.1 mm, 1.7 µm, Waters) and coupled to triple quadrupole mass spectrometer was used for UPLC/ESI-MS/MS analysis. Chromatographic separation was achieved using a gradient mobile phase of solvent A (acetonitrile) and solvent B (1% formic acid/aqueous solution) at a flow rate of 0.25 mL/min and a linear gradient from 10%A to 40%A for the first minute followed by isocratic elution with 40%A for 5 more minutes. Detection of 1-DNJ peak was carried out in positive electrospray ionization mode (ESI). Selective ion monitoring mode was used for quantification of 1-DNJ based on a standard calibration curve of 1-DNJ established under the same experimental conditions using concentrations of 1-50 µg/mL of standard 1-DNJ. The UPLC/ ESI-MS/MS
method was validated for linearity and reproducibility ($R^2=0.999$). Analysis of 1-DNJ in the extract was done in triplicate.

1.7. Determination of rutin content in BVLE

Rutin was selected as the chemical marker for standardization of BVLE extract. A calibration curve for rutin was established using 5 different concentrations (0.4-64 mg/mL). Serial dilutions of rutin and BVLE (100 µg/mL) were analyzed using the same conditions employed for the qualitative analysis of flavonoids as mentioned above.

1.8. Determination of α-glucosidase inhibitory activity

Assessment of α-glucosidase inhibitory activity was performed spectrophotometrically using 96-well microplate reader according to the method of Li et al. (2005). Acarbose was used as positive control and the assay was performed in triplicate. The results were expressed as the sample concentration required to inhibit 50% of the enzyme activity (IC$_{50}$).

1.9. Determination of radical-scavenging activity (DPPH assay)

The DPPH radical-scavenging assay was performed according to the method of Romano et al. (2009). All tests were performed in triplicate. Ascorbic acid was used as a positive control. Radical scavenging activity was calculated as EC$_{50}$ (effective concentration of the sample scavenging 50% of DPPH radical in methanol).
1.10. **In vivo** anti-diabetic activity

1.10.1. Animals

Adult male albino rats of Sprague Dawley strain (130-150 g) were used for assessment of the *in vivo* activity of the extracts. Animals were kept under controlled laboratory conditions for at least one week before starting the experiment and were allowed free access to water and fed a standard rat pellet diet composed of: vitamin mixture (1%), mineral mixture (4%), corn oil (10%), sucrose (20%), cellulose (0.2%), casein (10.5%) and starch (54.3%) during the entire experiment. All adopted procedures were in accordance with the guidelines of the EEC Directive 1986; 86/609/EEC for animal experiments and were approved by the Institutional Research Ethics Committee at the Faculty of Pharmacy, Cairo University, Egypt (REC-FOPCU, June 2013).

1.10.2. Determination of LD$_{50}$

Both BMLE and BVLE were dissolved in DMSO by sonication and administered by oral route to male albino rats (130-150 g) to determine their LD$_{50}$ adopting Karber's procedure (Karber 1931). The animals were divided into 6 groups, each of 4 rats. One of six doses (1-13.5 g/ kg b.wt.) was administered to each group and symptoms of toxicity and mortality rate were recorded 2, 24 and 48 hours after administration.

1.10.3. Experimental protocol

Type II diabetes was induced in overnight-fasted rats by a single intraperitoneal injection of freshly prepared STZ (50 mg/kg, in 0.1 M cold citrate buffer, pH 4.5). For the first two days after STZ injections, rats were given 5% glucose orally to avoid acute hypoglycemia induced by the elevated levels of serum insulin (Ercisli and Orhan 2007). Rats were tested for successful induction of diabetes at 2, 5 and 7 days after STZ injection. Blood samples were withdrawn from the tail vein and a blood glucose meter (GlucoDR: Dong-il Techno Town, Korea) was used to determine blood glucose levels. Only rats with blood glucose levels > 250 mg/dl were enrolled in the study. Healthy nondiabetic rats were used as normal control (group-I, 8 animals). Diabetic rats were divided into 6 groups (8 animals each) based on the treatment regimen as follows: group II, untreated diabetic; group III received a daily oral dose of gliclazide 15 mg/kg; groups IV and V received daily dose of 250 and 500 mg/kg B.W of BMLE, respectively and group VI and VII received daily dose of 250 and 500 mg/kg B.W of BVLE, respectively. After 4 weeks, animals were sacrificed under light anesthesia and blood was collected from the ophthalmic vein. Serum was separated by centrifugation and stored at -20°C for assessment of fasting blood glucose (FBG), insulin levels and liver and kidney biomarkers.

2. **Statistical analysis**

Experimental data were expressed as the mean ± standard deviation (SD). The statistical analysis was performed using the SPSS software (Version 20 for Windows, IBM, Chicago, IL).
Differences between groups were analyzed by one-way analysis of variation (ANOVA), followed by Dunnett’s test and the least significant difference (LSD) test. The difference with p-value <0.05 was considered to be significant.

References
Ercisli S. and Orhan E. Chemical composition of white (Morus alba), red (Morus rubra) and black (Morus nigra) mulberry fruits. Food Chem. 2007; 103 (4): 1380-1384.
Ivanova V, Marina S, Fabio C. Determination of the polyphenol contents in Macedonian grapes and wines by standardized spectrophotometric methods. J Serbian Chem Soc. 2010: 75 (1); 45-59.
### Table S1: Flavonoids and phenolic compounds in *M. nigra* and *B. variegata*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
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<tbody>
<tr>
<td></td>
<td><em>M. nigra</em></td>
</tr>
<tr>
<td>Total phenolics</td>
<td>65.44 ± 0.03 mg GAE/g</td>
</tr>
<tr>
<td>Total flavonoids</td>
<td>11.38 ± 0.007 mg QE/g</td>
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<tr>
<td>Quercetin</td>
<td>2.75 mg/g</td>
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<tr>
<td>Quercetin</td>
<td>1.6 mg/g</td>
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<tr>
<td>Hesperidin</td>
<td>1.35 mg/g</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.74 mg/g</td>
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<tr>
<td>Chlorogenic acid</td>
<td>2.0 mg/g</td>
</tr>
<tr>
<td>Ψ-Coumaric acid</td>
<td>0.34 mg/g</td>
</tr>
</tbody>
</table>
Figure S1. Fruiting branch of *Morus nigra* L.

Figure S2. Flowering branch of *Bauhinia variegata* L (B).

Figure S3: Effect of administration of BMLE on serum FBG and insulin levels
A, FBG levels measured by the end of each week of the four week study period in mg/dl. B, serum insulin levels measured in µIU/ml at the end of the study period.

Figure S4: Effect of administration of BVLE on serum FBG and insulin levels
A, FBG levels measured by the end of each week of the four week study period in mg/dl. B, serum insulin levels measured in µIU/ml at the end of the study period.

Figure S5: Effect of administration of BMLE on serum biomarkers for both liver and kidney functions.
A, serum level of liver enzymes AST and ALT in mg/dl. B, serum level of creatinine in mg/dl and C, serum level of urea in mg/dl as measured by the end of the study period.

Figure S6: Effect of administration of BVLE on serum biomarkers for both liver and kidney functions.
A, serum level of liver enzymes AST and ALT in mg/dl. B, serum level of creatinine in mg/dl and C, serum level of urea in mg/dl as measured by the end of the study period.
Figure S1

Figure S2
Figure S3
Figure S4
Figure S5

A

B

C

Normal cont  Diabetic cont  Gliclazide  250 mg/kg BMLE  500 mg/kg BMLE

Normal cont  Diabetic cont  Gliclazide  250 mg/kg BMLE  500 mg/kg BMLE

Normal cont  Diabetic cont  Gliclazide  250 mg/kg BMLE  500 mg/kg BMLE
Figure S6

A

B

C

Normal cont Diabetic cont Gliclazide 250 mg/kg BVLE 500 mg/kg BVLE Normal cont Diabetic cont Gliclazide 250 mg/kg BVLE 500 mg/kg BVLE Normal cont Diabetic cont Gliclazide 250 mg/kg BVLE 500 mg/kg BVLE