ABSTRACTS

Aim of Study: The aim was to investigate the hypoglycemic and anti-oxidant activities of the dried roots of *Morinda officinalis* in streptozotocin-induced diabetic rats.

Methodology: An ethanolic extract of the dried roots of *Morinda officinalis* and its three fractions (ethyl acetate, n-butanol and water) were obtained. We evaluated the hypoglycemic effects of three different single doses of the crude extract and its fractions in normal and diabetic rats for three hours after administration. Administration of the extract at 150 mg/kg twice daily for 10 days to the diabetic rats was also carried out. The effects of the 10-day treatment on the fasting serum glucose, insulin, total cholesterol, triglycerides, body weight, food intake, fluid intake, hepatic superoxide dismutase (SOD), catalase (CAT) activities, reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS) and renal TBARS levels were monitored.

Results: In the three-hour dose response study, the crude ethanolic extract reduced the fasting serum glucose levels of the diabetic rats significantly at 150 mg/kg but increased those of the normal rats significantly at 600 mg/kg only. The water fraction demonstrated a dose dependent hypoglycemic effect in the diabetic rats whereas the n-butanol fraction increased the fasting serum glucose levels of the diabetic rats significantly at 50 mg/kg only within three hours after administration. The 10-day oral administration of the extract reduced the fasting serum glucose, hepatic and renal TBARS level and significantly increased the hepatic SOD and CAT activities as well as GSH levels.

Conclusion: The results indicate that the dried roots of *Morinda officinalis* possess hypoglycemic, hyperglycemic and anti-oxidant properties.

Keywords: *Morinda officinalis*, diabetic rat, oxidative stress, hypoglycemic, anti-oxidant
and catalase (CAT) activities\(^\text{5}\). GSH, SOD and CAT are important anti-oxidant defenses of the body. It appears that the tissue anti-oxidant status may be an important factor in the development of diabetic complications.

The present study was undertaken to investigate possible hypoglycemic activity of the ethanolic extract of *Morinda officinalis* and its fractions in normal and STZ-induced diabetic rats. The evaluation of the possible anti-oxidant activity as well as the effects of chronic administration of the extract on the fasting serum glucose, insulin, total cholesterol and triglycerides levels in STZ-induced diabetic rats were also carried out.

**MATERIALS AND METHODS**

**Preparation of plant material**

Dried roots of *Morinda officinalis* were purchased from a local commercial source (Eu Yan Sang, Singapore). The roots (1 kg) were ground to powder and extracted with 80% denatured ethanol (20 L) till exhaustion at room temperature. After filtration with cotton wool, the filtrate was concentrated at 65°C by a rotavapor (Buchi Laborteknik AG, Postfach, Switzerland). The concentrate was then freeze-dried to yield 300 g of dark brown powder. The ethanolic extract was dissolved in distilled water before use.

Three fractions of the ethanolic extract were also prepared. Briefly, 100 g of the ethanolic extract were first partitioned into an ethyl acetate and water mixture. The water-soluble portion was further extracted with n-butanol. Subsequently, all the three portions were concentrated and freeze-dried before use. The yields of the ethyl acetate fraction, n-butanol fraction and water fraction were 1.1 g, 8.5 g and 69 g respectively.

**Animals**

Locally bred male Sprague-Dawley (SD) rats, aged 10 weeks (220-250 g body weight) were obtained from The Laboratory Animal Centre, National University of Singapore. The rats were housed in an air-conditioned room at 22 ± 1°C with a lighting schedule of 12 hours light (06.00 to 18.00) and 12 hours dark. A standard pellet diet (Glen Forest, WA, Australia) and tap water were supplied *ad libitum*.

**Induction of experimental diabetes**

Rats were fasted for 16 hours before the induction of diabetes with STZ (Sigma Chemical co., St Louis, MO, USA). Animals were injected intraperitoneally with 0.22-0.25 ml of a freshly prepared solution STZ (60 mg/ml in 0.01 M citrate buffer, pH 4.5) at a final dose of 60 mg/kg body weight. The diabetic state was assessed in STZ-treated rats by measuring the non-fasting serum glucose concentration 48 hours post STZ injection. Only rats with serum glucose levels greater than 300 mg/dl were used in experiments.

**EXPERIMENTAL PROCEDURE**

**Incremental dose finding experiment**

For the evaluation of the effects of the ethanolic extract on the fasting serum glucose in normal rats, the rats were fasted for 16 hours and divided into six groups of six animals each. Group 1, which served as the negative control group, received only a single oral dose of distilled water (5 ml/kg). Groups 2, 3, 4, 5 and 6 were treated with a single oral dose of 150, 300 and 600 mg/kg of extract, glibenclamide (10 mg/kg) (RBI Research Biochemical International, MA, USA) and metformin (500 mg/kg) (Pharmacy, National University of Singapore, Singapore) respectively. The blood samples were collected from the tail veins at 0 hour (just prior to the oral administration of the various treatments) and at 1 hour, 2 hours, 3 hours after administration of the treatments. The blood samples were centrifuged at 3,000 g for five minutes to obtain the serum for glucose assays. The same procedure was repeated in diabetic rats.

For the evaluation of the effects of the various fractions, the above procedure was carried out with Groups 2, 3 and 4 treated with each of the fractions instead of the extract. The three doses of the fractions, namely ethyl acetate, n-butanol and water used in the experiments were 50, 100 and 200 mg/kg. There were only four rats assigned to each group.

**Short term (10 days) repeated dosing (dosage as decided by the preceding dose finding experiments) study**

Diabetic rats (n=12) were randomly divided into three groups of four rats. Groups 1, 2 and 3 were treated with distilled water (5 ml/kg), metformin (500 mg/kg) and ethanolic extract of *Morinda officinalis* (150 mg/kg) respectively. The dose of extract was determined from the above incremental dose finding experiments evaluating the effects of 150, 300 and 600 mg/kg of extract on fasting serum glucose in normal and STZ-diabetic rats. On Day 1, blood samples were collected from the tail veins of the 16-hour fasted rats just prior to the initiation of the various treatment schedules. The rats were then dosed orally twice daily by gavage with either distilled water, metformin or extract for 10 days. Fasting serum glucose, insulin, total cholesterol and triglycerides levels of Day 1 were determined. Daily measurements of the body weight, food and fluid intakes of the rats were recorded. On the evening of Day 10, all rats were fasted for 16 hours and killed by decapitation the following morning. Blood was collected from the carotids and kept on ice. Kidneys and liver samples were frozen in liquid
nitrogen and immediately stored at -70°C for various assays. Serum was obtained by centrifuging the blood samples at 3,000 g for 15 minutes.

**Determination of serum glucose, insulin, total cholesterol and triglycerides**

Serum glucose concentrations were determined using a commercial diagnostic kit (Sigma Diagnostics, St Louis, MO, USA). Serum insulin concentrations were estimated using an enzyme-linked immunosorbent assay (ELISA) kit (Merckodia AB, Uppsala, Sweden) with rat insulin as standards. Serum total cholesterol and triglycerides concentrations were analysed using wet reagent diagnostic kits (Boehringer Mannheim, Germany).

**Anti-oxidative properties experiments**

**Thiobarbituric acid reactive substances (TBARS) measurement**

The TBARS levels measured as an index of malondialdehyde (MDA) production were determined by the method of Uchiyama and Mihara. MDA, an end product of lipid peroxidation reacts with thiobarbituric acid to form a red coloured complex. The measurement of MDA levels by thiobarbituric acid reactivity is the most widely used method for assessing lipid peroxidation. Briefly, 1 g of the liver and kidney samples were homogenised in 4 ml of 1.15% ice cold KCl using a Polytron homogeniser (Kinematica GmbH, Lucerne, Switzerland) to form a 25% (w/v) homogenate. To 0.1 ml of 25% homogenate, 0.2 ml of 8.1% dodecyl sodium sulphate salt (SDS), 1.5 ml of 1% phosphoric acid, 0.2 ml of distilled water and 1.0 ml of 0.6% 2-thiobarbituric acid were added. The mixture was heated in a boiling water bath for 45 minutes. Subsequently, the heated mixture was cooled in an ice bath, followed by an addition of 4.0 ml of n-butanol to extract the cold thiobarbituric acid reactants. The optical density of the n-butanol layer was determined at 353 nm after centrifugation at 1,000 g for five minutes and expressed as nmol MDA/25 mg wet weight.

**Superoxide dismutase (SOD) measurement**

SOD activity was measured based on the ability of the enzyme to inhibit the autoxidation process of pyrogallol. A modification of the procedure described by Marklund and Marklund was adopted for assay of SOD activity. Briefly, the tissues were homogenised in isotonic buffer (pH 7.4). The homogenate was centrifuged at 1,000 g for 10 minutes. 20 µl of 100-fold diluted tissue supernatant was added to 980 µl of the assay mixture containing 90 µl of 10 mmol/L of H₂O₂, 50 µl of Tris HCl buffer (pH 8.0) and 30 µl of distilled water. The rate of decomposition of H₂O₂ was monitored spectrophotometrically at 240 nm. SOD activity is expressed as k/mg protein, where k is the first order rate constant.

**Catalase (CAT) measurement**

CAT activity was measured based on the ability of the enzyme to break down H₂O₂. The method of Aebi was employed in the assay of CAT activity. Briefly, the tissues were homogenised in isotonic buffer (pH 7.4). The homogenate was centrifuged at 10,000 g for 20 minutes, then at 100,000 g for 60 minutes. To 0.5 ml of tissue supernatant, 1.5 ml of 0.2 mol/L Tris HCl buffer (pH 7.4). The homogenate was centrifuged sequentially at 10,000 g for 20 minutes, then at 100,000 g for 60 minutes. To 0.5 ml of tissue supernatant, 1.5 ml of 0.2 mol/L Tris HCl buffer (20 mmol/L EDTA, pH 8.2), 0.1 ml of 0.01 mol/L of 5,5'-dithiobiis-(2-nitrobenzoic acid) and 7.9 ml of methanol were added. The mixture was incubated at 37°C with occasional shaking for 30 minutes. The mixture was then centrifuged at 3,000 g 15 minutes and the absorbance of the supernatant was determined at 412 nm. The GSH concentrations of the samples were derived from the standard curve prepared using known amounts of GSH. GSH levels are expressed as µmol/mg protein.
Protein content measurement

Protein contents of the tissue supernatants were determined using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The results are expressed as means ± S.E.M. Data were analysed using unpaired Student's t-test. P values of <0.05 were considered to be statistically significant.

RESULTS

Incremental dose finding experiment

Effects of ethanolic extract on fasting serum glucose in normal and STZ-diabetic rats

As shown in Fig. 2, the fasting serum glucose concentrations were significantly higher in the extract-treated (600 mg/kg) normal rats when compared with the vehicle-treated normal rats at the first and second hour after oral administration (P<0.05, t-test). The extract (150 mg/kg) caused a significant reduction in the fasting serum glucose levels of diabetic rats throughout the three-hour study when compared with the vehicle (P<0.05, t-test). The other two doses of the extract however did not cause any significant changes in the glucose levels of the normal and diabetic rats when compared with the vehicle.

Effects of ethyl acetate fraction on fasting serum glucose in normal and STZ-diabetic rats

The ethyl acetate fraction did not produce any significant changes in the fasting serum glucose levels of the normal and diabetic rats when compared with the vehicle (Fig. 3).

Effects of n-butanol fraction on fasting serum glucose in normal and STZ-diabetic rats

It is shown in Fig. 4 that there was no significant difference in the fasting serum glucose concentrations between the n-butanol fraction-treated normal rats and the vehicle-treated normal rats. Oral administration of the fraction (50 mg/kg) produced a significant elevation of 23%, 36% and 31% from the 0 hour fasting serum glucose levels of the diabetic rats at the first, second and third hour respectively with the vehicle (P<0.05, t-test). The fasting serum glucose levels of the diabetic rats were however not affected by the other two doses of the fraction.

Effects of water fraction on fasting serum glucose in normal and STZ-diabetic rats

The effects of the water fraction are summarised in Fig. 5. The three different doses of the water fraction did not cause any significant changes in the fasting serum glucose levels of the normal rats

Table 1. Fasting serum glucose, insulin, total cholesterol and triglycerides levels in vehicle (distilled water), metformin and Morinda officinalis extract-treated STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Metformin (500 mg/kg)</th>
<th>Extract (150 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>Day 1</td>
<td>348 ± 41</td>
<td>381 ± 34</td>
</tr>
<tr>
<td></td>
<td>Day 11</td>
<td>393 ± 32</td>
<td>135 ± 32‡**</td>
</tr>
<tr>
<td>Insulin (µg/L)</td>
<td>Day 1</td>
<td>0.300 ± 0.037</td>
<td>0.293 ± 0.040</td>
</tr>
<tr>
<td></td>
<td>Day 11</td>
<td>0.161 ± 0.030</td>
<td>0.350 ± 0.068</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>Day 1</td>
<td>82 ± 4</td>
<td>73 ± 7</td>
</tr>
<tr>
<td></td>
<td>Day 11</td>
<td>77 ± 8</td>
<td>77 ± 7</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>Day 1</td>
<td>89 ± 10</td>
<td>110 ± 27</td>
</tr>
<tr>
<td></td>
<td>Day 11</td>
<td>147 ± 28</td>
<td>62 ± 16 *</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E.M. (n=4). * P<0.05, ** P<0.005, compared with the corresponding vehicle-treated group; ‡ P<0.005, compared with the corresponding Day 1 value.
when compared with the vehicle. Dose dependent hypoglycemic effects were however observed in the diabetic rats. The water fraction-treated (50 mg/kg) diabetic rats did not show any significant changes in the fasting serum glucose levels when compared with the vehicle-treated diabetic rats. The fasting serum glucose levels were significantly lower in the water fraction-treated (100 mg/kg) diabetic rats than those of the vehicle-treated diabetic rats at the second and third hour after administration (P<0.05, t-test). A significant reduction of 7%, 14% and 28% from the 0 hour fasting serum glucose level was observed in the 200 mg/kg extract-treated diabetic rats at the first, second and third hour respectively after oral administration when compared with the vehicle-treated diabetic rats (P<0.05, t-test).

Effects of glibenclamide, metformin on fasting serum glucose in normal and STZ-diabetic rats
It is shown in Fig. 2, 3, 4 and 5 that the glibenclamide-treated normal rats showed a significant reduction in the fasting serum glucose levels throughout the three-hour dose response study when compared with the vehicle-treated group. Metformin significantly reduced the fasting serum glucose levels of the diabetic rats at all the time points studied when compared with the vehicle. The glibenclamide-treated diabetic rats and metformin-treated normal rats showed no significant difference in the fasting serum glucose levels when compared with their respective vehicle-treated groups.

Short term (10-day) repeated dosing (dosage as decided by the preceding dose finding experiments) study Effects on fasting serum glucose, insulin, total cholesterol and triglycerides
The changes in the fasting serum glucose, insulin, total cholesterol and triglycerides are summarised in Table I. The fasting serum glucose levels were significantly lower in the extract- and the metformin-treated diabetic rats when compared with the vehicle-treated rats (P<0.05, t-test). No significant difference was observed in the insulin and total cholesterol levels between the three groups. Treatment with metformin led to significantly lower triglycerides levels when compared with the vehicle-treated group (P<0.05, t-test).

Effects on daily bodyweight, food and fluid intakes
The changes in bodyweight, food and fluid intakes were summarised in Fig. 6. Throughout the 10-day study, no significant difference was observed between the bodyweights of the vehicle-, extract- and metformin-treated diabetic rats. The extract did not produce any significant changes in the food and fluid intakes when compared with the vehicle. The food and fluid intakes were significantly lower in the metformin-treated diabetic rats when compared with the vehicle-treated group throughout the whole course of study.

Anti-oxidative properties experiments
Effects on hepatic and renal TBARS levels
The hepatic and renal TBARS levels were significantly lower in the extract- and metformin-treated diabetic rats than in the vehicle treated group (P<0.05, t-test) (Table II).

Effects on hepatic antioxidant enzymes and GSH
The hepatic SOD, CAT activities and GSH levels were significantly elevated in the extract- and metformin-
incremental dose finding experiment

The incremental dose finding experiments clearly demonstrated that the ethanolic extract of *Morinda officinalis* exhibits hypoglycemic, hyperglycemic and anti-oxidative properties in STZ-induced diabetic rats.

**DISCUSSION**

Our studies demonstrated that the ethanolic extract of *Morinda officinalis* exhibits hypoglycemic, hyperglycemic and anti-oxidative properties in STZ-induced diabetic rats.

**Table II. Malondialdehyde (MDA) content in liver and kidney of vehicle (distilled water), metformin and *Morinda officinalis* extract-treated STZ-induced diabetic rats.**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>TBARS value (nmol MDA/25 mg wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kidney</td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.80 ± 0.37</td>
</tr>
<tr>
<td>Metformin (500 mg/kg)</td>
<td>3.40 ± 0.17*</td>
</tr>
<tr>
<td>Extract (150 mg/kg)</td>
<td>2.99 ± 0.59*</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E.M. (n=4). * P<0.05 compared with the corresponding vehicle-treated group.

**Table III. Antioxidant status of liver in vehicle (distilled water), metformin and *Morinda officinalis* extract-treated STZ-induced diabetic rats.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Metformin (500 mg/kg)</th>
<th>Extract (150 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/mg protein)</td>
<td>21.62 ± 1.31</td>
<td>34.30 ± 1.91**</td>
<td>29.39 ± 2.42**</td>
</tr>
<tr>
<td>CAT (k/mg protein)</td>
<td>0.423 ± 0.020</td>
<td>0.610 ± 0.009**</td>
<td>0.530 ± 0.035**</td>
</tr>
<tr>
<td>GSH (µmol/mg protein)</td>
<td>0.166 ± 0.014</td>
<td>0.214 ± 0.010*</td>
<td>0.210 ± 0.009**</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E.M. (n=4). * P<0.05 compared with the corresponding vehicle-treated group. SOD, superoxide dismutase; CAT, catalase; K, first order rate constant; GSH, reduced glutathione. All assays were performed in duplicate at 25ºC.

**Fig. 4** Effects of vehicle (distilled water), n-butanol fraction of the ethanolic extract of *Morinda officinalis* (50 mg/kg, 100 mg/kg, 200 mg/kg), glibenclamide (10 mg/kg), metformin (500 mg/kg) on the fasting serum glucose levels in normal (A) and STZ-induced diabetic (B) rats. Each value represents mean ± S.E.M. (n=4). * P<0.05 compared with the corresponding vehicle-treated group. The % changes in fasting serum glucose at 1 hour, 2 hour and 3 hour were calculated from the corresponding 0 hour value (obtained just prior to the initiation of treatments) of each treatment group. The % changes in fasting serum glucose at 1 hour, 2 hour and 3 hour were calculated from the corresponding 0 hour value (obtained just prior to the initiation of treatments) of each treatment group.
metformin was effective in the diabetic rats, as it does not depend on a functional pancreas for its activity.

Our findings revealed that the water fraction exerts a similar but modest hypoglycemia effect when compared to metformin. Both the water fraction and metformin demonstrated a dose dependent hypoglycemic action in STZ-induced diabetic rats only. It is possible that the mechanism of the hypoglycemic action is similar to that of metformin. However further studies are required to validate the exact mechanism of the hypoglycemic action of the water fraction.

**Short term (10-day) repeated dosing (dosage as decided by the preceding dose finding experiments) study**

The dose of the extract (150 mg/kg) that was used in the 10-day study was found to be the optimum hypoglycemic dose in the earlier incremental dose finding study. Twice daily administration of the extract for 10 days significantly reduced the fasting serum glucose of diabetic rats without causing any significant changes in the insulin levels. This means that the extract does not trigger insulin secretion for its glucose-lowering action. Our findings on the effects of metformin on fasting serum glucose, insulin, total cholesterol and triglycerides concentrations after the 10-day treatment are in agreement with previous studies(7,15).

**Anti-oxidative properties experiments**

Studies have reported an increase in hepatic and renal TBARS concentrations in STZ-induced diabetic rats when compared with the normal rats(2,7,16). In diabetes, hypoinsulinaemia increases the activity of the enzyme, fatty acyl Coenzyme A oxidase, which initiates beta-oxidation of fatty acids, resulting in lipid peroxidation(1,2). Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors(5). Its products (lipid radical and lipid peroxide) are harmful to the cells in the body and are associated with atherosclerosis and brain damage(2). In the present study, the hepatic and renal TBARS levels were significantly lower in the extract-treated group compared to the vehicle-treated group. These findings suggest that the ethanolic extract may exert anti-oxidant activities and protect the tissues from lipid peroxidation.

Insulin deficiency in the diabetic state results in the impairment of glucose utilisation leading to an increased generation of oxygen free radicals(1,2). Studies have reported on the reduction of hepatic SOD and CAT activities in STZ-induced diabetic rats when compared with normal rats(7,17,18). SOD has been touted as one of the most important enzymes in the enzymatic anti-oxidant defence system. The superoxide anion has been known to inactivate CAT, which is involved in the detoxification of hydroxyl radical(5). SOD scavenges the superoxide anion to form hydrogen peroxide, hence diminishing the toxic effects caused by this radical. Wohlaib et al (1987) had suggested that the reactive oxygen free radicals could inactivate and reduce the hepatic SOD and CAT activities (17). In the present study, it was observed that the extract caused a significant increase in the hepatic SOD and CAT activities of the diabetic rats. This means that the extract can reduce reactive oxygen free radicals and improve the activities of the hepatic anti-oxidant enzymes.

Studies have shown that the hepatic GSH concentrations of STZ-induced diabetic rats are significantly lower when compared with the normal rats(7,19,20). GSH has a multifaceted role in anti-oxidant defence. It is a direct scavenger of free radicals as...
well as a co-substrate for peroxide detoxification by glutathione peroxidases (21). Loven et al (1986) had suggested that the decrease in hepatic GSH could be the result of decreased synthesis or increased degradation of GSH by oxidative stress in diabetes (19). In the present study, a significant elevation of hepatic GSH level was observed in the extract-treated diabetic rats. This indicates that the extract can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH, or have both effects.

Our findings that metformin increased the hepatic SOD, CAT activities, GSH levels and reduced the hepatic and renal TBARS levels in diabetic rats are consistent with the previous reports (2, 20). Zhang et al (2000) had suggested that these findings could be attributed to metformin’s insulin potentiation action and direct glucose lowering effect through interaction with insulin-independent regulatory mechanisms such as glucose transport (7). By improving the glucose utilisation processes, metformin reduces the generation of oxygen free radicals in the body, resulting in lesser oxidative damage to the tissues, and enhanced levels of anti-oxidant enzyme activities.

CONCLUSION
The present study shows that the ethanolic extract of Morinda officinalis not only possesses hypoglycemic and hyperglycemic properties but also reduces oxidative stress in diabetic rats. This study indicates that the dried roots of Morinda officinalis may not be useful for glycemic control in diabetic patients due to the presence of hyperglycemic compounds. However, its ability to reduce oxidative stress may help to prevent diabetic complications. Further studies should be undertaken to identify the active hypoglycemic compounds and investigate the mechanisms of hypoglycemic and anti-oxidant actions of the dried roots of Morinda officinalis.

ACKNOWLEDGEMENTS
The authors wish to thanks Annie Hsu, Peter Pushparaj and Zhang Xiang Fan for their excellent technical assistance. This study was supported by the Departmet of Pharmacology, National University of Singapore.

REFERENCES


