Summary

The aim of the present study was to evaluate antihyperglycemic activity of methanol extract of Citrus maxima leaf (MECM) in streptozotocin (STZ; 65 mg/kg b.w.) induced diabetic rats. Three days after STZ induction, diabetic rats received MECM orally at a dose of 200 and 400 mg kg\(^{-1}\) body weight daily for 15 days. Glibenclamide (0.5 mg kg\(^{-1}\) p.o.) was used as a reference drug. Blood glucose levels were measured on days 0, 4, 8 and 15. Serum biochemical parameters viz. SGOT, SGPT and ALP were estimated. The TBARS and GSH levels of the pancreas, kidney and liver were determined. MECM significantly (P<0.001) and in a dose dependent manner normalized blood glucose levels, serum biochemical parameters, decreased lipid peroxidation, and recovered GSH as compared to those of STZ control. The present study inferred that in STZ-induced diabetic Wistar rats, C. maxima leaf demonstrated a potential antihyperglycemic effect that might be attributed to its antioxidant property.

Introduction

Diabetes mellitus (DM) is an endocrine-metabolic disorder of increasing incidence and clinical relevance, contributing to high morbidity and mortality rates (1). Due to population aging, urbanization, increased prevalence of obesity and physical inactivity, the number of individuals affected by DM is increasing in many parts of the world (2). In view of this growing incidence, the study of the physiological routes of DM becomes crucial for the emergence of novel therapeutic procedures (3). Type 2 DM is a chronic metabolic disorder characterized by abnormalities in carbohydrate and lipid metabolism leading to postprandial and fasting hyperglycemia, dyslipidemia and hyperinsulinemia. Insulin resistance is considered a significant pathogenic factor in type 2 diabetes and an obvious target for antidiabetic medication (4). Diabetes is usually associated with an increased production of the molecules of reactive oxygen species (ROS) and/or impaired antioxidant defense systems, which result in oxidative damage leading to ROS mediated diabetic pathogenesis.
Disturbances of the antioxidant defense system in diabetes involve enhancement of lipid peroxidation, alteration in antioxidant enzymes and impaired glutathione metabolism (5).

Oxidative stress is the imbalance between the generation of ROS and the body defense mechanisms. Environmental pollutants, toxic habits (drugs, smoking, and/or alcohol), inadequate nutrition, excess solar radiation, large exposure to toxic substances (fertilizers and pesticides), drug metabolism (side effects), and a high physical or psychical stress are the most common exogenous factors originating ROS in the human body (6). Oxidative stress has also been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiovascular disorders, neurologic disorders, as well as in the process of aging (7).

Citrus (C.) maxima (J. Burm.) Merr. (Rutaceae) is known as shaddock or pomelo in English and Batabilebu in Bengali. C. maxima is widely distributed throughout India. The pulp of C. maxima is stated to possess appetizer, antitoxic, cardiac stimulant, and stomach tonic properties, as reported in ancient and medieval literature (8).

The major flavanones of C. maxima are neohesperidin and naringin, which are higher in the seed than in unripe fruits (9), and its extract exhibited antioxidant activity through free radical-scavenging in vitro and reduced ROS in H$_2$O$_2$-treated HepG2 cells (10). DPPH free radical-scavenging activity and ferric-reducing antioxidant power values determined for the essential oil were 26.1±1.2% and 2.3±0.3 mM, respectively, which were significantly higher than those of various fruit pulp extracts (11). Hesperidin, naringin, caffeic, p-coumaric, ferulic and vanillic acid are present in the fruit juice (12). Hesperidin and naringin are beneficial for improving hyperlipidemia and hyperglycemia in type 2 diabetic animals by partly regulating the fatty acid and cholesterol metabolism and affecting the gene expression of glucose-regulating enzymes (13). The present study aimed to explore the antihyperglycemic potential of C. maxima leaf against streptozotocin (STZ)-induced diabetes in rats.

**MATERIAL AND METHODS**

**Plant material**

C. maxima (J. Burm.) Merr. (Rutaceae) leaves were collected from Nadia region of West Bengal in the months of March-April, 2007. The plant material was authenticated by the Botanical Survey of India, Shibpur, Howrah; and the voucher specimen (PMU-1/JU/2007) is stored in our laboratory for further references.

**Preparation of the extract**

The leaves of C. maxima were shade-dried and powdered by mechanical grinder. About 500 g of the plant material was successively extracted with petroleum ether and methanol in a Soxhlet apparatus. The methanol was then evaporated under reduced pressure to get dry extract in vacuo (at 35 °C and 0.8 MPa) in a Buchi evaporator, R-114 (MECM, yield 8.1%). The dry extract was kept in a vacuum desiccator until use. Preliminary phytochemical analysis and chromatographic studies of MECM revealed the presence of alkaloids, steroids, flavonoids and saponins (14).

**Drugs and chemicals**

Glibenclamide tablet (Daonil™, Hoechst, India), streptozotocin (Sigma Aldrich, St. Louis, USA), 5,5-dithio bis-2-nitro benzoic acid (DTNB), reduced glutathione (Sisco Research Lab, Mumbai, India), thiobarbituric acid (TBA), trichloroacetic acid (TCA) and nitroblue tetrazolium (NBT) were obtained from Merck. All other reagents used were of AR grade and obtained commercially.

**Animals**

Six- to eight-week-old male Wistar albino rats (180±20 g) were obtained from B. N. Ghosh and Co., Kolkata. The animals were kept at 25±2 °C and relative humidity of 40%-45%, with alternative 12-h day and night cycles. All animals were acclimatized to
identical laboratory conditions for seven days prior to
the study. The animals had free access to pellet food
(Hindustan Lever, Mumbai, India) and water.

**Acute toxicity**

Acute toxicity of the extract was determined
according to the OECD guideline No. 420 (15). Male
albino mice weighing 27-30 g were used for this study.
MECM was given to four groups (n=5) of animals at
5, 50, 300 and 2000 mg kg\(^{-1}\) b.w., p.o. The treated
animals were observed up to 14 days for mortality and
general behavior.

**Oral hypoglycemic activity in normal rats**

Healthy male Wistar albino rats weighing 180±20 g
were selected for the study. The animals were divided
into three groups (n=6). Group I served as normal
saline control (5 mL kg\(^{-1}\) b.w., 0.9% NaCl p.o.);
groups II and III received MECM at doses of 200 and
400 mg kg\(^{-1}\) b.w., p.o., respectively. Blood samples
were collected from the tail tip at 0 (before oral
administration) and 2 h 30 min after drug
administration (16). The blood sugar level was
measured using Accu-chek Active test strip in an
Accu-chek Active Test meter.

**Induction of experimental diabetes**

Streptozotocin (65 mg kg\(^{-1}\) b.w. i.p.) was
administered to overnight fasted male Wistar albino
rats (180±20 g) and observed for 5 days (17). After 5
days, blood glucose level was measured in overnight
fasted rats by using Accu-chek Active test strip in an
Accu-chek Active Test meter. The animals that
exhibited blood glucose level of more than 250 mg dL\(^{-1}\)
were selected for the study.

**Treatment of diabetic animals**

Twenty-four male diabetic rats and six normal rats
(180±20 g) were divided into five groups (n=6). Group
I served as nondiabetic saline control. Group II served
as diabetic control. Groups III and IV received MECM
(200 and 400 mg kg\(^{-1}\) b.w., p.o., respectively), and
group V received reference drug glibenclamide (0.5
mg kg\(^{-1}\) b.w., p.o.) daily for 15 days (18).

**Sample collection and analysis**

Fasting blood sugar in study animals was determined
on days 0, 5, 8 and 15 day in samples collected from
tail vein. On day 15, blood was collected from the
retro-orbital plexus of the eye for determination of
SGPT, SGOT (19) and ALP (20).

All animals from different groups were sacrificed by
cervical dislocation on day 15 and the liver, kidney
and pancreas were isolated for estimation of thiobarbituric
acid reactive substances (TBARS) and reduced
 glutathione (GSH).

TBARS were estimated in the liver, kidney and
pancreas by the method of Fraga et al. (21) and are
expressed as mmol 100 g\(^{-1}\) of tissue. GSH was
determined by the method of Ellman (22) and GSH
activity is expressed as mg 100 g\(^{-1}\) of liver tissue.

**Statistical analysis**

All values of body weight, fasting blood sugar and
biochemical parameters were expressed as mean ±
standard error of mean (SEM) and were analyzed by
one-way ANOVA followed by Dunnett’s \(t\) test. The
values of \(P<0.001\) were considered statistically very
significant and \(P<0.05\) significant. GraphPad Prism
version 5.0 was used for statistical calculations.

**RESULTS**

**Acute toxicity**

The acute toxicity study carried out in Swiss albino
mice showed that MECM was safe up to 2000 mg kg\(^{-1}\)
b.w., p.o. No death was observed until the end of the
study.

**Hypoglycemic activity in normal rats**

The effects of MECM on lowering blood sugar levels
in normal rats are summarized in Figure 1. The
MECM treated groups exhibited significant reduction
in the blood sugar levels after 2.5 h in a dose dependent manner when compared with saline control group.

**Fasting blood glucose (FBG) levels**

The measured blood glucose levels of nondiabetic and STZ induced diabetic rats are shown in Figure 2. The administration of MECM to diabetic rats produced a significant reduction in blood glucose levels in a dose dependent manner as compared with STZ control group.

**Effect on body weight**

Body weights of normal and diabetic rats are summarized in Figure 3. Final body weights were significantly decreased in STZ control group when compared with the saline control group. The observed data indicated improvement of body weight after treatment with the extract when compared to STZ control group.

**Serum biochemical parameters**

Biochemical parameters such as SGPT, SGOT and ALP were restored to normal levels after MECM were treatment as compared with diabetic control group (Fig. 4).
Pancreatic, renal and hepatic biochemical parameters

The levels of TBARS and GSH activities in pancreatic, renal and hepatic tissues of experimental diabetic rats are shown in Figure 5. There was a significant elevation of lipid peroxide in the pancreas, liver and kidney with diabetes in diabetic control group as compared to the saline control group. It was seen that the administration of MECM decreased the pancreatic, hepatic and renal TBARS levels, which is an indication of the inhibition of oxidative damage in these tissues. There was also a significant decrease in GSH level in STZ control group when compared with the saline control group. The administration of MECM at doses of 200 and 400 mg kg\(^{-1}\) b.w. increased the GSH content in the liver, kidney and pancreas of STZ induced diabetic rats.

DISCUSSION

Streptozotocin is widely used for the induction of diabetes mellitus in experimental animals. It is postulated to induce diabetes by degeneration and necrosis of β-cells of the islets of Langerhans of pancreas, which leads to reduction in insulin release (23). The ability of STZ to selectively enter β-cells via the low affinity glucose transporter GLUT2 in the plasma membrane can be attributed to the presence of glucose moiety in it. After STZ enters the cells, it gets converted to reactive methylcarbonium ions that alkylate DNA and induce free radical generation, which target the DNA sugar moiety and result in DNA strand breakage (24). In the present investigation, it was observed that the administration of MECM at doses of 200 and 400 mg kg\(^{-1}\) b.w. produced effective hypoglycemic and antihyperglycemic activity in normoglycemic as well as STZ induced diabetic rats. MECM also showed a significant controlling effect in the loss of body weight that is normally encountered in diabetic rats.

Elevated levels of the serum enzymes SGPT, SGOT and ALP in diabetic control group reflected significant alteration of liver function by STZ induction. Treatment with MECM restored the elevated enzyme levels to normal values in a dose dependent manner.

Under normal physiological conditions, human body can compensate for a mild degree of oxidative stress and remove oxidatively damaged molecules by activating antioxidant enzymes. These antioxidants are able to resist oxidative stress by scavenging free radicals, inhibiting lipid peroxidation, and increasing glutathione (25).

The TBARS and GSH levels of the pancreas, kidney and liver were examined for the effect on augmented oxidative stress in diabetic animals. Lipid peroxidation is a natural phenomenon involved in peroxidative loss in unsaturated lipids, thus bringing about lipid degradation and membrane disorganization. Peroxidized lipid has been considered to play a significant role in the pathogenesis of several diseases, and may be taken as a molecular mechanism of cell injury under pathological conditions. Lipid peroxidation is usually measured through its catabolite.
malondialdehyde (MDA) in terms of TBARS as a marker of oxidative stress (26). In the present study, it was observed that there was a significant decrease in the TBARS levels of the MECM treated rats in comparison with diabetic (STZ) control group.

Glutathione is one of the abundant tripeptide nonenzymatic biological antioxidants present in the liver and kidney. GSH plays a multifunctional role in antioxidant defense. It is a direct scavenger of free radicals as well as a co-substrate for peroxide detoxification by glutathione peroxidase (27). It is postulated that a decrease in tissue GSH could be the result of either decreased synthesis or increased degradation of GSH by oxidative stress (28). In our study, it was observed that MECM was able to decrease elevated levels of GSH in comparison with diabetic (STZ) control rats.

The present study showed the methanol extract of *C. maxima* leaves to exert a marked antihyperglycemic effect in STZ induced diabetic Wistar rats as well as hypoglycemic activity in normoglycemic rats. The *C. maxima* plants are rich in flavonoids, which are polyphenolic compounds having potent antioxidant property. *In vitro* free radical scavenging activity of MECM was performed by the authors and the extract was found to have significant free radical scavenging activity when tested against different free radical models (29). The potential antihyperglycemic action of MECM is plausibly due to its underlying antioxidant property.

As already discussed in the Introduction section, *C. maxima* fruit contains flavonoids viz. neohesperidin, naringin (9) and hesperidin (12). Both hesperidin and naringin are proven to be hypoglycemic agents and their hypoglycemic activity is postulated to be partly mediated by hepatic glucose-regulating enzymes in C57BL/KsJ-db/db mice (30). Naringin also lowers plasma lipids and enhances erythrocyte antioxidant enzyme activities in hypercholesterolemic subjects (31). Hesperidin has protective action against diabetes induced brain damage in rats (32). Dietary hesperidin also exerts hypoglycemic and hypolipidemic effects in STZ induced diabetic rats (33). Naringin provided significant amelioration of hypoglycemic and antioxidant activity in STZ induced diabetic rats (34). Preliminary phytochemical analysis of MECM indicated the presence of alkaloids, flavonoids, steroids, etc. Flavonoids are polyphenolic compounds that are well-known antioxidants because of their electron-donating properties, either scavenging the principal propagating radicals or halting the radical chain.

Therefore, a hypothesis can be drawn from the present study that hesperidin, naringin and related flavonoids may be responsible for the antihyperglycemic activity of *C. maxima* leaf. Hence, it can be postulated that further investigation is required to pinpoint the biologically active principle of *C. maxima* leaf that may provide a novel, safe antidiabetic agent.

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