Sub-chronic oral administration of the ethanolic extracts of dried Terminalia chebula mature fruits in streptozotocin (STZ)-induced type 2 diabetes mellitus (T2DM) model of Long-Evans (L-E) rats improve glycemic, lipidemic and anti-oxidative status

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INTRODUCTION
Diabetes mellitus is possibly the world’s largest growing metabolic disorder, and as the knowledge on the heterogeneity of this disorder is advanced, the need for more appropriate therapy increases (Bailey et al., 1986). Oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus by continuous formation of abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms by the process of glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. These consequences of oxidative stress can promote the development of complications of diabetes mellitus (Maritim et al., 2003). Terminalia chebula is a common herbaceous plant in Southern Asia region and every part of the tree has been used in the preparation of many ayurvedic medicines for household remedy against various human ailments, from antiquity. Both the fresh and dried fruits of the T. chebula are very popular traditional medicines for the treatment of diabetes, it is also reported to have antioxidant and free radical scavenging activities (Cheng et al., 2003). Although a number of studies have screened the fruits of T. chebula for hypoglycemic activities, but most were on acute T2DM animal model; in-depth studies on the possible biochemical mechanism

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ABSTRACT
Mechanisms of the long known anti-diabetic effects exerted by the fruits of Terminalia chebula are not yet clearly understood. Here, we sought to get a biochemical view of the mechanisms. First, a single injection of streptozotocin (STZ) was given (50 mg/Kg of body weight, bw) to 48 hours old pups of Long-Evans (L-E) rats intraperitoneally (ip) to induce type 2 diabetes (T2DM). At the age of 3 months, a total of twenty male L-E rats, having T2DM were included in this study and divided into 4 groups (n = 5, for each group). T2DM was confirmed by a standard oral glucose tolerance test (OGTT). Next, T2DM rats were orally administered with a single dose of 80% ethanolic extracts of either the fresh or dried fruits for consecutive 28 days. Water and glibenclamide were used as negative and positive control, respectively. Administration of dried (p = 0.001) and fresh (p = 0.02) fruit extracts significantly reduced the fasting serum glucose level as compared to that of water control. Fasting serum lipid profile show that administration of both fresh and dried fruit extracts caused a significant reduction of triglycerides (p = 0.016), total cholesterol (p = 0.001) and low density lipoprotein-cholesterol (p = 0.001). Examinations of antioxidant potential profile demonstrate that dried fruit extracts only decreased the levels of plasma MDA in T2DM rats to a notable extent (62%). Although the fruit extracts could not improve the levels of serum insulin, but increased the liver glycogen content to a remarkable extent (240%). This study indicates that sub-chronic administration of the ethanolic extracts of dried Terminalia chebula mature fruits improve glycemic, lipidemic and anti-oxidative status in T2DM male L-E rats and that the hypoglycemic action may be mediated by an extra-pancreatic mechanism.

Abbreviations: STZ= Streptozotocin, T2DM=Type 2 Diabetes Mellitus, OGTT=Oral glucose tolerance test, TG=Triglyceride, HDL-C=Low density lipoprotein cholesterol, LDL-C=Low density lipoprotein cholesterol, FSG = Fasting serum glucose, Glib=Glibenclamide, WC = Water control, TCFF=Ethanolic extract of T. chebula fresh fruit type 2 group, TCDF=Ethanolic extract of T. chebula dry fruit type 2 group, SPSS= Statistical Package for Social Science, ANOVA= Analysis of variance.

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(s) of action of the *T. chebula* fruits in chronic animal model of T2DM are relatively lacking. Thus the aim of the study was to understand the possible biochemical basis of antidiabetic effects of mature fruits of *T. chebula* on T2DM model rats.

**MATERIALS AND METHODS**

**Study Site**

The study was conducted in the Department of Pharmacology, Biomedical Research Group (BMRG), BIRDEM, Dhaka, Bangladesh.

**Plant Materials**

In this study, both the mature fresh and dried fruits of *T. chebula* were used.

The fresh fruits were collected from *T. chebula* trees in the Jahangirnagar University campus area, Savar, Dhaka. The plant was identified by the Bangladesh National Herbarium, Dhaka (DACB Accession Number: 35370). The dried fruits were collected from the commercially available sources of Savar market.

**Preparation of Extracts**

In the laboratory, both the mature fresh and dried fruits were washed thoroughly after collection and dried. After that the fruits were ground. Finally, these ground portions were extracted separately using 80% ethanolic solvent.

These extracts were filtered with thin and clean cloth and then filtered by filter paper.

Following the completion of extraction and filtration both the mature extracts, prepared from fresh and dried fruits were concentrated under reduced pressure using a rotary evaporator (BUCHI R-114, Switzerland) maintained at 55ºC. The semi-dried ethanolic extracts were further dried in a freeze drier (HETOSICC, Heto Lab Equipment, Denmark) at -55ºC temperature and stored in a reagent bottle at -8ºC in a freezer.

**Preparation of type 2 diabetes model rats**

Type 2 diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) in citrate buffer(10ml, pH 4.5), at a dose of 90mg/kg of body weight (Bonner-Weir et al., 1981) into the 48 hours old rat pups with average weight 7 g. The pups were allowed to grow at a constant room temperature of 22±5ºC, 40-70% humidity conditions and the natural day-night cycle with an ad libitum access to food at the BIRDEM animal house.

The rats had no access to food during the whole period of blood sampling. The influence of circadian rhythms was avoided by starting all experiments at 7:30 a.m. Following 3 months of STZ injection, male long Evans rats weighing 190-260 g were picked out to examine their blood glucose level by oral glucose tolerance test (OGTT). Diabetic model rats with blood glucose level >7.00 mmol/L at fasting condition were selected for studying the effects of *T. chebula* extracts.

**Experimental design**

A total of 20 type 2 diabetic model rats were used in this 28 days experimental period. They were divided into 4 groups:

- **Group-1** (n=5): Type 2 water control group [10 ml water/kg body weight]
- **Group-2** (n=5): Type 2 glibenclamide group [5 mg/10 ml (9.9 ml H2O + 0.1 ml Tween 20)/kg body weight]
- **Group-3** (n=5): Ethanolic extract of *T. chebula* fresh fruits treated group (1.25g/kg body weight)
- **Group-4** (n=5): Ethanolic extract of *T. chebula* dried fruits treated group (1.25g/kg body weight)

Rats of all groups were kept under similar environmental conditions, and were provided with enough food and water throughout the experiment. The body weight of the animals was measured weekly.

**Sub chronic study**

Water, glibenclamide and extracts of *T. chebula* fresh and dried fruits were fed to the corresponding rat groups at the given dose for 28 consecutive days.

Fasting serum glucose test was performed on the 0, 14th and 28th day of the study.

Blood samples were collected from tail tips to measure serum glucose, total cholesterol, triglyceride, HDL, LDL, serum insulin, hemoglobin, plasma malondialdehyde (MDA) levels and reduced glutathione (GSH) concentration in erythrocyte. On the 28th day, after the animals were decapitated, their blood was collected from heart and liver was collected to estimate liver glycogen.

**Biochemical analysis**

The following parameters of type 2 diabetic model rats were measured for the anti-diabetic and antioxidant effects of *T. chebula*.

Serum glucose was measured by Glucose Oxidase (GOD-PAP) method using micro-plate reader (Bio-Tec, ELISA).

The total cholesterol was measured by enzymatic colorimetric (Cholesterol Oxidase/ Peroxidase, CHOD-PAP) method (Randox Laboratories Ltd., UK), using autoanalyzer, AutoLab; serum triglyceride (TG) by enzymatic colorimetric (GPO-PAP) method (Randox Laboratories Ltd., UK) using autoanalyzer, AutoLab. Serum insulin by Rat Insulin enzyme linked immunosorbent assay (ELISA) method (Crystal Chem Inc., USA).

The absorbance was measured by microplate ELISA Reader (Bio-Tek EL-340, USA). Liver glycogen levels were estimated by Anthrone-sulphuric acid method (Vries, 1954). Estimation of MDA level by using the thiobarbituric acid reactive substances (TBARS) method (Yagi, 1994, cited by Ates et al., 2004). Reduced glutathione was assayed by Ellman’s method (Ellman, 1959).
Chronic Rat Model of Type -2 Diabetic Mellitus (T2DM)

Recording of body weight on 0, 7th, 14th, 21st and 28th days

Statistical Analysis

Data from the experiments were analyzed using the Statistical Package for Social Science (SPSS) software for windows version 12 (SPSS Inc., Chicago, Illinois, USA). All the data were expressed as Mean ± SD or as Median (Range) as appropriate. Statistical analysis of the results was performed by using the student’s t-test (paired and unpaired), ANOVA (analysis of variance) followed by Bonferroni and Dunnett post hoc test and Mann Whitney (u) test. The limit of significance was set at p<0.05.

RESULTS

Effect of T. chebula extracts on glucose homeostasis

Results of FSG level of the studied rats at baseline (before onset of feeding i.e., 0 day), 14th day and 28th day of feeding is presented in Table 1.

It was found that on 14th and 28th day the FSG level of the glibenclamide, ethanolic extract of T. chebula fresh fruits (TCFF) and dried fruits (TCDF) treated groups decreased gradually. Type-2 rats, fed with dried fruit extract showed a better ameliorated diabetic condition (p=0.001) than the glibenclamide treated groups (p=0.002) both in 14th and 28th day.

TCFF treated group showed significant reduction of serum glucose level at the 28th day [FSG mmol/l (M±SD) 7.27±0.97 on 0 day vs. 5.61±0.86 on 28th day; p=0.02].

Table 1: Effect of T. chebula extract on fasting serum glucose level of type-2 diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose (mmol/L) 0 day</th>
<th>Glucose (mmol/L) 14 day</th>
<th>Glucose (mmol/L) 28 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Control</td>
<td>7.95±1.25</td>
<td>7.48±1.08</td>
<td>7.49±0.25</td>
</tr>
<tr>
<td>Glibenclamide treated group</td>
<td>9.13±1.62</td>
<td>6.89±0.85**</td>
<td>4.43±1.09**</td>
</tr>
<tr>
<td>Fresh fruit Ethanol extract</td>
<td>9.02±1.59</td>
<td>7.27±0.97</td>
<td>5.61±0.86*</td>
</tr>
<tr>
<td>Dried fruit Ethanol extract</td>
<td>8.34±0.51</td>
<td>7.88±0.55**</td>
<td>5.12±1.24**</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ±SD. Statistical analysis between group comparison was done by using one way ANOVA with post hoc Bonferroni test.*=p<0.05; **=p<0.005

Effects of T. chebula extracts on lipid profile of type 2 diabetic model rats

Table 2 shows the effects of T. chebula extracts on the lipid profile of type-2 diabetic model rats.

It was found that in type-2 water control group, the level of serum cholesterol increased at the end of the study period [serum cholesterol (M±SD) mg/dl (62±7) 0 day vs (75±9) 28 day]. Glibenclamide treated rats showed a significant decrease in the
total serum cholesterol level after 28 days feeding by 13% [serum cholesterol (M±SD mg/dl (53±6) 0 day vs (46±5) 28 day (p=0.001). In case of both the fresh and dried fruit extracts of T. chebula, there were accordingly 14% and 16% reduction, when compared with 0 day value. Reduction of total serum cholesterol by both the extracts was significant (p=0.001). In case of the TCFF and TCDF extract groups, total serum cholesterol level decreased by 14% and 16%, respectively, in comparison to the 0 day values. Therefore, this result indicates that T. chebula had a reducing effect on total serum cholesterol level after sub-chronic feeding. Serum TG level increased by 17% in type-2 water control group [serum TG (M±SD mg/dl (59±15) vs. significant (69±17) 28 day). Favorable effects on serum TG level were found with glibenclamide, TCFF (p=0.016) and TCDF (p=0.016) treated groups after 28 day Serum TG level decreased by the influence of glibenclamide, TCFF and TCDF by 21%, 33% and 17%, respectively. In addition, it was found that in water control group, serum HDL-C level decreased by 16% and LDL-C level was increased by 79% after the 28 day study period. In glibenclamide treated group, HDL-C level was decreased by 19% and LDL-C level’s increased by 25%. HDL-C level in TCFF group remain unchanged, while showed 19% reduction in TCDF treated group. However, in case of LDL-C, both the TCFF (p=0.001) and TCDF (p=0.001) treated group showed significant reduction. The obtained results indicated that T. chebula might be helpful in correcting dyslipidemia that occurs in diabetes.

Effects of T. chebula extracts on the serum insulin of type-2 diabetic model rats.

Sub-chronic effect of T. chebula extracts on the serum insulin of type-2 diabetic model rats is shown in Table-3. It is clearly seen from the Table that at 28th day, glibenclamide raised insulin level by 15%. The rest of the groups showed a reduction in serum insulin level although the reduction was not statistically significant.

Effects of T. chebula extracts on the liver glycogen of type-2 diabetic model rats.

The effects of T. chebula extracts on hepatic glycogen content of type 2 diabetic model rats are presented in Table 4. It is clear from the Table that in comparison to control, hepatic glycogen content among the test groups was increased after 28 days of chronic oral administration of fruits. Serum glycogen content as 2.56±1.61 (M ±SD, mg/g) in T2DM water control group. Following treatment with glibenclamide, TCFF and TCDF for 28 days increase in hepatic glycogen content was not significant. Hepatic glycogen content raised by 113%, 41% and 140% in glibenclamide, TCFF and TCDF groups, respectively.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cholesterol (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>HDL</th>
<th>LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Day</td>
<td>28 Day</td>
<td>0 Day</td>
<td>28 Day</td>
</tr>
<tr>
<td>Water Control (n=5)</td>
<td>62±7</td>
<td>75±9</td>
<td>59±15</td>
<td>69±17</td>
</tr>
<tr>
<td>Glibenclamide (n=5)</td>
<td>(100%)</td>
<td>(121%)</td>
<td>(100%)</td>
<td>(117%)</td>
</tr>
<tr>
<td>Fresh fruit Ethanol extract (n=5)</td>
<td>53±6</td>
<td>46±5**</td>
<td>61±17</td>
<td>48±15</td>
</tr>
<tr>
<td>Dried fruit Ethanol extract (n=5)</td>
<td>(100%)</td>
<td>(87%)</td>
<td>(100%)</td>
<td>(79%)</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ±SD. Statistical analysis between group comparison was done by using one way ANOVA with post hoc Bonferroni test. *= p<0.05; **= p<0.005.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Insulin (ng/dl) 0 Day</th>
<th>Insulin (ng/dl) 28 Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Control (n=5)</td>
<td>0.43±0.09 (100%)</td>
<td>0.23±0.12 (53%)</td>
</tr>
<tr>
<td>Glibenclamide (n=5)</td>
<td>0.46±0.002 (100%)</td>
<td>0.53±0.02 (115%)</td>
</tr>
<tr>
<td>Fresh fruit Ethanol extract (n=5)</td>
<td>0.56±0.20 (100%)</td>
<td>0.28±0.16 (50%)</td>
</tr>
<tr>
<td>Dried fruit Ethanol extract (n=5)</td>
<td>0.66±0.33 (100%)</td>
<td>0.55±0.62 (83%)</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ±SD. Statistical analysis between group comparison was done by using one way ANOVA with post hoc Bonferroni test.

Effects of T. chebula extracts on body weight of T2DM model rats

Effect of T. chebula extracts on body weight of type-2 rats is presented in figure 2. It is seen that body weight was decreased in all groups although the reduction was not statistically significant.
Effects of *T. chebula* extracts on the malondialdehyde (MDA) and reduced glutathione (GSH) of type-2 diabetic model rats.

The concentrations of MDA and GSH are shown in Table-5. After 28 days study period as expected, type-2 diabetic water control rats showed the highest MDA level 1.99 μmol/ml, when compared with other groups. TCDF administration to type-2 rats significantly lowered serum MDA level compared to control group [p<0.04 in Mann-Whitney U test]. In T2DM water control group, erythrocyte GSH conc. was 12.91±1.64 (M±SD), mg/g Hb. No significant change was found in the erythrocyte GSH level in any group. Erythrocyte GSH level was 12.02±2.82 (Glc), 10.70±2.31 (TCFF) and 12.42±3.16 (TCDF).

**Table 5**: Effects of *T. chebula* extracts on serum malondialdehyde (MDA) of type-2 diabetic model rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (μmol/ml)</th>
<th>GSH (mg/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Control (n=5)</td>
<td>1.99±0.34</td>
<td>12.91±1.64</td>
</tr>
<tr>
<td>Glibenclamide (n=5)</td>
<td>1.72±0.47</td>
<td>12.02±2.82</td>
</tr>
<tr>
<td>Fresh fruit Ethanol extract (n=5)</td>
<td>1.50±0.74</td>
<td>10.70±2.31</td>
</tr>
<tr>
<td>Dried fruit Ethanol extract (n=5)</td>
<td>1.24±0.56</td>
<td>12.42±3.16</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ±SD Statistical analysis between group comparison was done by using Mann-Whitney U Test (MDA) one way ANOVA with posthoc Bonferroni test (GSH).

**DISCUSSION**

The development of insulin resistance, β-cell dysfunction, impaired glucose tolerance seen in T2DM as well as the progression of its long-term complications including microvascular and macrovascular dysfunction results from the oxidative stress, through the production of reactive oxygen species (ROS) (Wright *et al.*, 2006).

To date, major pharmacogenetic studies have focused on response to sulfonylureas, biguanides, and TZDs (Distefano *et al.*, 2010). However, prominent side-effects and enormous costs of such modern medicines are the main reason for an increasing number of people seeking alternative plant based herbal drugs or botanicals that may have less severe or no side-effects for the management of diabetes. (Srinivasa, 2007). The study of traditional plant based medicines might offer a natural key to unlock a diabetologist’s pharmacy for the future (Nalamolu *et al.*, 2006).

*T. chebula* is widely used as a traditional medicine by diabetic patients in Indian subcontinent. Fruits of this plant have been demonstrated to possess several medicinal values, such as hypoglycemic (Murali *et al.*, 2007), hypolipidemic (Khanna *et al.*, 1993), antiabetic (Gandhipuram *et al.*, 2006) and antioxidant (Subramaniyan *et al.*, 2005) activities. The presence in *T. chebula* of biologically active ingredients such as gallic acid, chebulic acid, 1,6-di-O-galloyl-β-D-glucose, punicalagin, 3,4,6-tri-O-galloyl-β-D-glucose, casuarinin, chebulanin, corilagin, neochebulinic acid, terchebulin, ellagic acid, chebulagic acid, chebulinic acid, and 1,2,3,4,6-penta-O-galloyl-β-D-glucose might be responsible for its medicinal properties (Juang *et al.*, 2004). Based on the multipurpose use of the mature fresh and dried fruits of *T. chebula*, the present study has been conducted to evaluate the anti-diabetic and anti-oxidant effect of the mature fresh and dried fruits of *Terminalia chebula* using STZ induced diabetic rats.

In the present study streptozotocin (STZ-well known for its selective pancreatic islet β-cell cytotoxicity) induced diabetic rats were chosen as the animal model because it resembles many of the features of human diabetes mellitus (Tomlinson *et al.*, 1992). Glibenclamide is often used as a standard anti-diabetic drug in STZ induced diabetes to compare the efficacy of variety of hypoglycemic compound (Pradesh *et al.*, 2001). It also exhibits a moderate anti-oxidant activity. Therefore in this study, this drug serves as positive control.

The results of the present study showed that the oral administration of *T. chebula* extract for 28 days gradually and significantly decreased the levels of serum glucose and lipid profile. The effects of *T. chebula* on diabetic complications associated with dyslipidemia were assessed by measuring the atherogenic lipids (i.e., cholesterol and triglycerides) after sub-chronic feeding of *T. chebula* extracts to diabetic rats. The obtained results demonstrated significant reduction of cholesterol, TG and LDL level at the end of 28 day study period. Thus fruits of *T. chebula* are anti-hyperlipidemic agents (Dipa *et al.*, 2010).

The STZ induced experimental diabetic model rats, used in this study were hypoinsulinemic. Fasting insulin level in normal rats (long Evan strain) ranges usually 3 ng/ml (Dwright, 2006). In our experimental group of diabetic rats had 2-3 folds less content of insulin (Type-2 WC=0.43±0.09 ng/dl). Only the glibenclamide treated group increased serum insulin level. The 4 weeks treatment with *T. chebula* did not improve serum insulin level in type-2 diabetic rats. Therefore, our finding revealed that slowly generated hypoglycemic effect of *T. chebula* on STZ induced type-2 rats may involve an extra-pancreatic effect, since serum insulin level did not increase.

Liver glycogen level may be considered as the best marker for assessing hypoglycemic activity of any drug. This indicates that peripheral free glucose is being stored in the liver in the form of glycogen by increasing glycogenesis. Increase liver glycogen level was observed in *T. chebula* treated group, especially in the dried fruit extract group in type 2 model rats. Therefore, it may be ascertained that the hypoglycemic activity of *T. chebula* in type 2 model rats is due to increased uptake of glucose for the formation of glycogen by enhanced glycogenesis. This may be one of the probable mechanisms for the hypoglycemic action. There is a clearly documented link between diabetic complications and lipid peroxidation. Hypoinsulinemia increases the activity of the enzyme, fatty acyl-CoA oxidase that initiates β-oxidation of fatty acids. This results in lipid peroxidation (Horie *et al.*, 1981), which is determined by thiobarbituric acid (TBAR) substances level. In one study, it has been found that in STZ induced diabetic rats, tissue blood MDA levels increased due to lipid peroxidation. In our experiment, the MDA level was decreased when treated with both the extracts of *T. chebula* and in case of TCDF it showed a significant reduction of the MDA level (p<0.04) and this value was much better when compared with the group treated with glibenclamide. Depletion of reduced glutathione (GSH) either by conjugation and removal from the cell or oxidation to oxidized glutathione could...
significantly affect the overall redox potential of the cell (Hansen et al., 2001). Our result showed a decrease in the GSH level, when treated with the extracts. In the 28 day study period, *T. chebula* did not show any significant change in the body weight compared with glibenclamide and water control group. In conclusion, ethanolic extract of *T. chebula* fruits has potential antihyperglycemic action on STZ induced diabetic rats and the effect has been found to be complementary with the action of glibenclamide. The hypoglycemic effect is probably mediated by extra-pancreatic action of *T. chebula*. It also possesses moderate antioxidative property as it is rich in polyphenolic compounds. Therefore, treatment with *T. chebula* provides a rationale for the use of *T. chebula* in Ayurvedic medicinal treatment.

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**REFERENCES**


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