Original Article

Antioxidant and Hypoglycemic Effects of *Tithonia diversifolia* Aqueous Leaves Extract in Alloxan-induced Diabetic Mice

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**ABSTRACT**

Diabetes is a chronic disease cause the metabolic disorder and associated with serious complications. Various studies have shown that diabetes mellitus is associated with increased the formation of free radicals and decreased antioxidant potential. Oxidative stress is increased in diabetes because of multiple factors. Dominant among these factors is glucose autoxidation leading to the production of free radicals. Treatment with the antioxidants may play an important role in the improvement of diabetes. In this study, we aimed to determine the antioxidant and anti-hyperglycemic properties of *Tithonia diversifolia* aqueous leaves extract (TDA). The potential antioxidant was examined and the results showed that the total phenolic content and the total antioxidant capacity of TDA were 55.92 ± 4.45 GAE mg/g dry weight and 93.09 ± 37.91 µM TEAC/mg dry weight, respectively. The anti-hyperglycemic activity of TDA at dose 500 mg/kg BW were studied by oral glucose tolerance test (OGTT) in normal mice and were daily orally administrated in alloxan-induced diabetic mice for 4 weeks. Hypoglycemic effect of TDA at dose 500 mg/kg BW showed significantly reduced blood glucose level on OGTT in normal mice (P<0.05). Moreover, TDA treated alloxan-induced diabetes mice for 30 days significantly decreased blood levels of glucose, total cholesterol, triglyceride and low density lipoprotein-cholesterol (LDL-cholesterol) and increased high density lipoprotein-cholesterol (HDL-cholesterol). The malondialdehyde (MDA), the lipid peroxide product of oxidative marker, was improved in TDA-treated group. These results suggested that TDA exerts its antioxidant property by inhibiting the chain reaction of lipid peroxidation and acts as the hypoglycemic activity, reducing the blood glucose level in alloxan-induced diabetes mice. Toxicity and the mechanism of TDA should further study and development as dietary supplements for the treatment of type 2 diabetes.

**Key words:** *Tithonia diversifolia* leaf, Antioxidant, Hypoglycemia, Diabetes mellitus

**Introduction**

Diabetes mellitus (DM) is a metabolic disease characterized by chronic hyperglycemia resulting from defects in insulin metabolism and impaired function in carbohydrate, lipid and protein metabolism that lead to long-term complications such as kidney, eye, nerves, heart and blood vessel damage. Diabetes mellitus is divided into two main groups namely, type 1 diabetes is an absolute deficiency of insulin secretion which develop and identified by serological evidence of an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers, and type II which results from insulin resistance and a gradual beta-cell dysfunction. The World Health Organization (WHO) estimates that more than 346 million people worldwide have diabetes mellitus. Without intervention, this number is likely to increase more than twofold by 2030 [50] The number of adults (aged ≥ 20) with diabetes in the world is estimated to increase by 122%, from 135 million in 1995 to 300 million in 2025 [18]. Oxidative stress results from an imbalance between radical-generating and radical-scavenging systems that are, increased free radical production or
reduced activity of antioxidant defenses or both these phenomena [34]. Various studies have shown that diabetes mellitus is associated with increased formation of free radicals and decrease in antioxidant potential which plays a significant in part in the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance, and type 2 diabetes mellitus [44,29,14,45]. In diabetes, protein glycation and glucose autoxidation may generate free radicals, which in turn catalyse lipid peroxidation associated with tissue damage, and cell death, leading to increased free radical production and compromised free radical inhibitory and scavenger systems, which further exacerbate the oxidative stress and diabetes complication [7,13].

The potential of the antioxidant constituents of traditional plant medicines for the maintenance of health and protection from diabetes is also raising interest among patients and scientists. Therefore, an investigation of such agents from traditional medicinal plants have become particularly important. Tithonia diversifolia (Hems1) A. Gray (TD) is an impressive member of the sunflower family, Asteraceae commonly known as Mexican sunflower. The power of TD was submerged into boiling distilled water for 15 min. The solution was filtered, centrifuged, frozen at -20°C and then lyophilized. TD aqueous extract (TDA) was stored at -20°C until used.

**Collection and preparation of plant extracts:**

The leaves of *Tithonia diversifolia* (TD) plant were collected from Yala Province in Southern part of Thailand. The leaves of TD were washed and dried in hot air oven at 50°C. The power of TD was submerged into boiling distilled water for 15 min. The solution was filtered, centrifuged, frozen at -20°C and then lyophilized. TD aqueous extract (TDA) was stored at -20°C until used.

**Determination of total phenolic content:**

Total phenolic content of TDA was quantified using the Folin-Ciocalteu’s method, with gallic acid as a standard. Twenty microliter of freshly prepared TDA was added to 1.55 ml of double deionized water and 100µl Folin-Ciocalteu reagent thoroughly mixed and incubated for 5 min at room temperature. Following incubation, 300 µl of the Na2CO3 (2%, W/V) solution was added and the mixture was allowed to stand at room temperature for an additional 2 h and the absorbance was measured at 765 nm. The total phenolic content was expressed as gallic acid equivalents of TDA (GAE mg/g TDA). All determinations were performed three times.

**ABTS cation radical scavenging activity:**

The total antioxidant activity of TDA was measured by the ABTS radical cation decolorization assay (Pellegrini et al., 2002). Briefly, the mixture solution of 5 ml of 7 mM ABTS and 80 µl of 140 mM potassium persulphate was allowed to stand in the dark at room temperature for 12–16 h before used. Then, 1 ml of ABTS was added to glass test tube containing 50 µl of TDA and mixed by vortex mixer for 30 s. The absorbance was measured at 734 nm after 2 min. The percentage of radical scavenging activity was calculated by comparing the absorbance values of control without the extract. All determinations were performed three times.

**Animals:**

Adult male ICR mice with body weights of 25 to 30 g were obtained from National laboratory animal center, Mahidol University. The mice were fed on a pellet diet and water *ad libitum*. The animals were maintained on a 12 h light/dark cycle in a controlled environment under standard conditions of temperature and humidity. All animal experimental procedures were approved by the Animal Care and Use Committee of Walailak University.

**Oral Glucose Tolerance Test (OGTT):**

After fasting for 12 h, normal mice were divided into 3 groups with six mice in each group.

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**Materials and Methods**

**Chemicals:**

All chemicals and reagent kits used in this study were obtained from Sigma Company (St Louis, USA).
Group I: received distilled water, to serve as controls
Group II: received TDA (500 mg/kg BW, orally)
Group III: received metformin (60 mg/kg BW, orally), to serve as a standard drug

After administration of distilled water, TDA and metformin for 30 min, glucose (2.0 g/kg BW) was administrated orally to these mice. Blood glucose levels were measured from the tail vein at 0, 30, 60, 120 and 180 min after the glucose was loaded.

Experimental induction of diabetes in mice and lipid metabolism:

The mice were injected with alloxan monohydrate dissolved in sterile normal saline at a dose of 120 mg/kg BW. After the 5th day of injection, mice displaying glycosuria, hyperglycemia (with a blood glucose of 200-300 mg/dl) and decreased body weight were used for the experiment.

Experimental design:

Overnight fasted mice were divided into six groups as follows:

- Group 1: Normal mice treated with distilled water
- Group 2: Diabetic mice treated with distilled water
- Group 3: Diabetic mice treated with TDA (100 mg/kg BW)
- Group 4: Diabetic mice treated with TDA (250 mg/kg BW)
- Group 5: Diabetic mice treated with TDA (500 mg/kg BW)
- Group 6: Diabetic mice treated with glibenclamide (60 mg/kg BW)

A total of 36 mice (30 diabetic surviving mice and 6 normal mice) was used and they were randomly divided into six groups with eight mice per group. Blood sample was collected from tail vein of all the groups at 0, 14 and 28 day. At the end of 4 week, animals were anesthetized using Nembutal sodium solution (65 mg/kg BW) and blood was obtained via left ventricle puncture into the heparin tubes and were centrifuged at 3,000 rpm for 10 min. The plasma sample was analyzed for blood glucose, total cholesterol, triglycerides, low density lipoprotein-cholesterol (LDL-cholesterol) and high density lipoprotein-cholesterol (HDL-cholesterol) using automatic chemistry analyzer (KoneLab 20, Tokyo, Japan). The liver and pancreas tissues were collected, homogenated in cold phosphate saline and determined the levels malondialdehyde (MDA).

Determination of Lipid peroxide (malondialdehyde, MDA):

Malondialdehyde (MDA) was used as a measure of lipid peroxidation [40]. Briefly, 200 µl samples of brain homogenate were mixed with trichloroacetic acid (1:1) and centrifuged at 3000 rpm for 15 min. Then 15% thiobarbituric acid was added to the supernatant, heated at 100 °C in boiling water bath for 30 min, and centrifuged. The supernatant were measured with spectrophotometer at 595 nm. The lipid peroxide levels were compared with a standard curve constructed from MDA. The results were expressed as nmoles/mg protein.

Statistical analysis:

All data are presented as mean ± SEM for sixth mice in each group. Comparison between group by one-way analysis of variance (ANOVA) followed with the Student-t-test. The Newman-Keuls Multiple Comparisons Test was utilized to analyze the statistical significance of the data from experimental and control groups, P-values < 0.05. All statistical analyses were performed using SPSS.

Results:

Total phenolic content and total antioxidant activity:

The amount of total phenolic and the antioxidant activity of TDA were determined as Table 1. The results showed that the total phenolic content and the total antioxidant of TDA were 55.92 ± 4.45 GAE mg/g dry weight and 93.09 ± 37.91 µM TEAC/mg dry weight, respectively.

Effect of TDA on Oral Glucose Tolerance Test (OGTT):

Plasma glucose (FPG) levels were markedly elevated, in TDA (500 mg/kg BW) mouse treated group on OGTT at 30, 60, 120 and 180 min showed hypoglycemia rebound effect observed when compared with the control group. Treatment with the diabetic drug metformin (60 mg/kg BW) presented a reduction of glucose level at 30 and 60 min followed by subsequent fall up to 120 minutes. A significant (P<0.05) reduction in blood glucose level was observed in the group treated with TDA (500 mg/kg BW) (Fig 1).

Anti-diabetic effect of TDA:

In animals treated with alloxan a significant increase in blood glucose level was observed on 7 days when compared to the normal mouse control collected, homogenated in cold phosphate saline and determined the levels malondialdehyde (MDA).
administration of diabetic drug glibenclamide (60 mg/kg BW) showed a significant decreased in blood glucose level when compared to the diabetic control group (P<0.05) (Fig. 2).

**Anti-hyperlipidemia effect of TDA:**

In addition to change in glucose homeostasis, diabetes leads to change in blood lipid profile. Alloxan induced diabetes mice resulted significant (P<0.05) elevation of total cholesterol, triglyceride, LDL-cholesterol where as HDL-cholesterol levels were decreased when compared to the normal mouse control group. TDA and glibenclamide were significantly (P<0.05) reduced total cholesterol, triglyceride, LDL-cholesterol while HDL-cholesterol levels were increased when compared to diabetic control group (Fig 3-6).

**Lipid peroxide (malondialdehyde, MDA):**

Malondialdehyde (MDA), one of the end products of lipid peroxidation has become the principal and the most studied product of polyunsaturated fatty acid peroxidation [7]. The MDA levels in liver and pancreas tissues in alloxan-induced diabetes group at the end of the study period were significantly higher than normal mice control groups (P<0.05, Fig. 7a, 7b). In TDA and glibenclamide treatment groups, MDA level in liver and pancreas tissues significantly reduced (P<0.05) compared to diabetes control group at the end of the study period (Fig 7a, 7b).

**Discussion:**

The present study, the aqueous leaves extract of *Tithonia diversifolia* (TDA) was shown a significant antihyperglycemia effect on tolerance of glucose (OGTT). The TDA showed a marked hypoglycemia, hypolipidemia effects in alloxan-induced diabetic mice were observed in long-term treatment as the dose dependent manner. The TDA treatment in diabetes mice evidently reduced the level of lipid peroxide (malondialdehyde, MDA), the lipid peroxidation product indicator of oxidative damage of pancreas and liver tissues.

In diabetes, hyperglycemia causes increased production of reactive oxygen species (ROS), for all tissues from glucose auto-oxidation and protein glycolylation [36]. Diabetes produced disturbances of lipid profile, especially an increased susceptibility to lipid peroxide, which is responsible for incidence of atherosclerosis, the major complication of diabetes mellitus [11]. Enhanced oxidative stress and changes in antioxidant capacity were observed in both clinical and experimental diabetes mellitus [26]. Decreased in antioxidant potential which play a central role in the complications of diabetes [1] indication that antioxidants play a major role in protecting against molecular oxidative damage [9,10]. Traditional plant with high level and strong antioxidant compounds have an important role in improvement of disorder involving oxidative stress such as diabetes mellitus. The present study showed that TDA contained total phenolic content which correlated to the total antioxidant capacity. Its function may play a role as free radical scavengers in the diabetic which known to be related of hyperglycemia and oxidative stress [39]. Scavenging reactive oxygen species have previously observed to improve the quality of life [27,3] in diabetic patients by preventing macrovascular and microvascular complications [24,42].

Antioxidants protect β-cells from oxidation by inhibiting the lipid peroxidation chain reaction and thus they play an important role in the diabetes. Plants containing natural antioxidants such as tannins, flavonoids, vitamin C and E can preserve β-cell function and prevent diabetes induced ROS formation lead to inhibit lipid peroxidation. The ability of the phenolic compounds depend on the redox properties of their phenolic hydroxyl groups, that allow them to act as reducing agents, hydrogen-donating antioxidants and oxygen quenchers [34]. In case of the phenolic components of TDA, our data have been reported the potential in total antioxidant activity and reduced the lipid peroxidation product (MDA) in alloxan-induced diabetes mice as found in the flavonoids treatment can regenerate the damaged β-cell in diabetic mice [12].

The Oral Glucose Tolerance Test (OGTT) measures the body’s ability to use glucose, the main source of energy. OGTT can be used to diagnose pre-diabetic and diabetes. The TDA showed ability to reduce the elevated glucose level in normal mice better than the diabetic drug metformin, which the mechanism of its action includes; direct stimulation of glycolysis in tissue with increase glucose removal from blood, slowing of glucose absorption from the gastrointestinal tract with increased glucose to lactate conversion by enterocytes and reduction of plasma glucagon levels.

Alloxan is the most commonly employed agent for the induction of diabetes in experimental animal model. There is increasing evidence that alloxan causes diabetes by rapid depletion of β cells by DNA alkylation and accumulation of cytotoxic free radicals that is suggested to result from initial islet inflammation, followed by infiltration of activated macrophages and lymphocyte in the inflammatory focus. It leads to a reduction in plasma insulin concentration leading to a stable hyperglycemia state [41]. Alloxan induced diabetic rats with more than 200 mg/dl of blood glucose level were considered to be diabetic and used for the study. Administration of TDA (500 mg/kg BW) for 30 days showed significant effect on FBG reduction observed in alloxan induced diabetic mice compared to diabetic mice control group (Fig 2). Moreover, the
concentration of TDA (500 mg/kg BW) which exerted the hypoglycemic observed in the alloxan-induced diabetic mice was better improvement than glibenclamide.

Previously, the potential of medicinal plants have been reported the effects on glucose metabolism through the various mechanisms, including, play a function role as insulin-like substances [30], inhibition the insulinase activity [2], increased insulin secretion from β-cells of islets of langerhans or its release from bound insulin [4,6] and may increase cells in the pancreas by activating regeneration of pancreatic cells [5]. The mechanism of TDA on the hypoglycemic properties will be further study.

Hyperlipidemia is well known associated with hyperglycemia in diabetic mice. The abnormalities of glucose metabolism was shown the correlated in lipid metabolism as evidenced from the elevation of serum total cholesterol, triglyceride, LDL-cholesterol, and reduction of HDL-cholesterol. In diabetes, hypoinsulinaemia increase the activity of enzyme fatty acyl-coenzyme A oxidase, which initiates β oxidation of fatty acids, resulting in lipid peroxidation [19] as found significant elevated of MDA level in liver and pancreas of diabetic mice in this study. The orally administration of TDA for 30 days showed a reduction in the levels of total cholesterol, triglyceride, LDL-cholesterol, where as HDL-cholesterol was increased compared to diabetic control. Treatment with TDA and glibenclamide significantly reduced MDA level in liver and pancreas tissues. The level of lipid peroxidation and reactive oxygen spices (superoxide anion, hydrogenperoxide and hydroxyl radical) are common marker oxidative stress in diabetes. Lipid peroxidation refers to the oxidative degradation of lipid that impairs cell membrane functions resulting the cell damage and leading to several pathologies and cytotoxicity [27]. These results indicate that TDA ameliorated glucose and lipid metabolism and also prevented lipid peroxidation, the oxidative damage marker, in diabetic mice.

<table>
<thead>
<tr>
<th>Total phenolic compound (mg GAE/g dry weight)</th>
<th>Total antioxidant capacity (µM TEAC/mg dry weight)</th>
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<tr>
<td>55.92 ± 4.45</td>
<td>93.09 ± 37.91</td>
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Mean ± SEM values of three independent determinations. GAE; Gallic acid equivalent, TEAC; Tralox equivalent antioxidant capacity.

![Fig. 1: Effects of Tithonia diversifolia aqueous leaves extract (TDA) on glucose tolerance test (OGTT) in normal fasted mice. Results are present as mean ± SEM, n=6 and three separated experiments. *P<0.05, significant difference between TDA treatment and distilled water (DW) control group. **P<0.05, TD treatment compared to metformin group.](image-url)
Fig. 2: Effect of TDA on blood glucose levels in alloxan-induced diabetics mice. Results are present as mean ± SEM, n=6. *P<0.05 compared to normal mice. **P<0.05 compared to diabetic control mice (DM). TDA100, 250 and 500; *Tithonia diversifolia* aqueous leaves extract (TDA) 100, 250 and 500 mg/kg BW, respectively. GLB; Glibenclamide 60 mg/kg BW.

Fig. 3: Effect of TDA on blood total cholesterol levels in alloxan-induced diabetics mice. Results are present as mean ± SEM, n=6. *P<0.05 compared to normal mice. **P<0.05 compared to diabetic control mice (DM). TDA100, 250 and 500; *Tithonia diversifolia* aqueous leaves extract (TDA) 100, 250 and 500 mg/kg BW, respectively. GLB; Glibenclamide 60 mg/kg BW.

Fig. 4: Effect of TDA on blood triglyceride levels in alloxan-induced diabetics mice. Results are present as mean ± SEM, n=6. *P<0.05 compared to normal mice. **P<0.05 compared to diabetic control mice (DM). TDA100, 250 and 500; *Tithonia diversifolia* aqueous leaves extract (TDA) 100, 250 and 500 mg/kg BW, respectively. GLB; Glibenclamide 60 mg/kg BW.
Fig. 5: Effect of TDA on blood LDL-cholesterol levels in alloxan-induced diabetics mice. Results are present as mean ± SEM, n=6. *P<0.05 compared to normal mice. ** P<0.05 compared to diabetic control mice (DM). TDA100, 250 and 500; *Tithonia diversifolia* aqueous leaves extract (TDA) 100, 250 and 500 mg/kg BW, respectively. GLB; Glibenclamide 60 mg/kg BW.

Fig. 6: Effect of TDA on blood HDL-cholesterol levels in alloxan-induced diabetics mice. Results are present as mean ± SEM, n=6. *P<0.05 compared to normal mice. ** P<0.05 compared to diabetic control mice (DM). TDA100, 250 and 500; *Tithonia diversifolia* aqueous leaves extract (TDA) 100, 250 and 500 mg/kg BW, respectively. GLB; Glibenclamide 60 mg/kg BW.

Fig. 7a: Effect of TDA on lipid peroxidation levels in liver tissues in alloxan-induced diabetics mice. Results are present as mean ± SEM, n=6. *P<0.05 compared to normal mice. ** P<0.05 compared to diabetic control mice (DM). TDA100, 250 and 500; *Tithonia diversifolia* aqueous leaves extract (TDA) 100, 250 and 500 mg/kg BW, respectively. GLB; Glibenclamide 60 mg/kg BW.
Fig. 7b: Effect of TDA on lipid peroxidation levels in pancreas tissues in alloxan-induced diabetics mice. Results are present as mean ± SEM, n=6. *P<0.05 compared to normal mice. ** P<0.05 compared to diabetic control mice (DM). TDA100, 250 and 500; *Titonia diversifolia* aqueous leaves extract (TDA) 100, 250 and 500 mg/kg BW, respectively. GLB; Glibenclamide 60 mg/kg BW.

Conclusion:

The results from this study have confirmed that the TDA is an interesting source of antioxidant, improves glucose metabolism and reduced the elevation of lipid profile and lipid peroxidation. Further studies are progressing in mechanism of TDA on the reduction of blood glucose and development the dietary supplement product for the treatment of type 2 diabetes.

Acknowledgement

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References


