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Effect of *Tithonia diversifolia* Leaf Extract on Leptin, Adiponectin, and Insulin Receptor Levels in Diabetic Rats

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ABSTRACT: This study aimed to evaluate the effect of *Tithonia diversifolia* extract (TDE) on leptin, adiponectin, and insulin receptor (IR) concentrations in diabetic rats. Twenty-four Wistar rats were divided into a control and treatment groups (n=6 per group). The control group received normal saline, and the treatment groups received 0.25% sodium carboxymethyl cellulose, TDE at 100 mg/kg body weight (bw), and catechin at 10 mg/kg bw for 7 days. On day 8, the rats were sacrificed, blood samples were obtained, and leptin, adiponectin, and insulin concentrations were measured using avidin-horseradish peroxidase sandwich-enzyme-linked immunosorbent assay. A calorimetric method was used to measure blood glucose (BG) and total serum cholesterol concentrations. The pancreas and kidneys were collected for the measurement of renal IR and macrophage cluster of differentiation (CD)14 levels using immunohistochemical staining. Acute type 2 diabetes mellitus (T2DM) with elevated BG and total serum cholesterol concentrations was observed in the treatment groups administered streptozotocin. The administration of TDE at 100 mg/kg bw significantly decreased leptin and increased adiponectin concentrations ($P \leq 0.05$). Furthermore, TDE treatment significantly increased renal IR and decreased macrophage CD14 levels ($P<0.05$). Therefore, TDE decreased leptin and BG concentrations by increasing IR levels. TDE also suppressed the necrosis of pancreatic tissues by inhibiting macrophage CD14 expression in diabetic rats. However, further research is necessary to determine the effect of TDE on interleukin and IR levels in the related tissues of patients with T2DM.

Keywords: CD14, diabetes mellitus, insulin receptor, *Tithonia diversifolia*

INTRODUCTION

Type 2 diabetes mellitus (T2DM), a metabolic syndrome associated with a chronic increase in blood glucose (BG) concentrations, is caused by genetic factors, lifestyles, and obesity. Insulin resistance and impaired insulin secretion are also typical features of T2DM (Balbaa et al., 2016).

The global prevalence of diabetes mellitus in adults increased by 463 million (6.20%) in 2020 (International Diabetes Federation, 2019). In addition, diabetes mellitus was responsible for 6.70 million deaths in 2021. This means there is 1 death every 5 seconds. It increases the risk of diseases such as heart attack, stroke, blindness, nephropathy, and gangrene (World Health Organization, 2016). More than 80 million people are estimated to have diabetes mellitus in Southeast Asia and the Western Pacific (Asia-Pacific region) countries (Lee et al., 2007). For example, in Indonesia, the prevalence of diabetes mellitus increased by 2% from 2013 to 2018 (Ministry of Health of Indonesia, 2018). In Surabaya, a large city in Indonesia and the capital of East Java Province, diabetes mellitus is the disease with the fourth highest mortality rate (Ministry of Health of Indonesia, 2017). It contributes to the development of cardiovascular disease risk, and obesity is the major contributing factor to cardiovascular disease and diabetes mellitus. An increased leptin concentration in patients with obesity reduces the response of pancreatic β-cell, resulting in increased insulin secretion and likely leading to hyperinsulinemia. Leptin regulates BG concentrations by increasing insulin sensitivity of liver and muscle cells in normal conditions (Majewska et al., 2016).

Generally, the leptin : adiponectin ratio is significantly associated with the incidence of diabetes mellitus among

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teenagers with obesity (Maahs et al., 2009). Leptin and adiponectin are produced in the adipose tissue and are involved in regulating insulin sensitivity and inflammation (Safai et al., 2015). Adiponectin has antidiabetic functions, strengthens insulin signaling pathways, reduces glucose production in the liver, increases fatty acid oxidation, improves mitochondrial function, and regulates glucose metabolism in the liver and muscles (López-Jaramillo et al., 2014; Chakraborti, 2015). Meanwhile, synthetic drugs are used to decrease BG concentrations in patients with diabetes mellitus; however, these drugs have many side effects on human health. As traditional medicine using medicinal plants is an alternative for disease prevention and treatment (Hughes et al., 2012), people have used natural remedies for diabetes. One of the plants with medicinal potential is *Tithonia diversifolia*, which is traditionally used to prevent stomach pain, bloating, diarrhea, and inflammation. *T. diversifolia* leaves, roots, stems, fruits, and seeds are sources of chemicals. For example, *T. diversifolia* leaves contain active substances (phytochemicals), such as alkaloids, saponins, saponin glycosides, tannins, balsam, and volatile oils (John-Dewole and Oni, 2013). *T. diversifolia* also has antidiabetic potential; it is commonly known as the insulin plant among Indonesians (Mabou Tagne et al., 2018). In addition, *T. diversifolia* is used in antidiabetic treatments in China (Zhao et al., 2012). A critical component in the pathogenesis of diabetes is insulin.

Insulin, an important hormone produced by pancreatic β-cells, regulates glucose homeostasis in various tissues. The mechanism of action of insulin includes inhibiting gluconeogenesis, stimulating glycogen synthesis, and activating lipogenesis in the liver (Hirako, 2016). In muscle and fat tissues, insulin induces glucose uptake through the insulin receptor (IR) activation pathway. The IR substrate is activated through the activation of phosphoinositide 3-kinase and phosphoinositide-dependent kinase-1 for protein synthesis, cell development, transcription, and glycogen synthesis. Insulin also plays a role in regulating cell growth and proliferation (Samarghandian et al., 2017).

Cluster of differentiation (CD)14 macrophages are phagocytic cells in the innate immune system that facilitate the binding of lipopolysaccharide (LPS) to Toll-like receptor-4 (TLR-4). The binding of LPS-CD14 complex to TLR-4 activates the inflammatory signaling pathway by activating nuclear factor kappa B, the protein kinase cascade by mitogen induction, and activated T cell nucleus factor. In addition, the *CD14* gene is expressed by myeloid cells and macrophages on hepatocytes and adipocytes. These cellular macrophages can be active in inflammatory processes caused by obesity and T2DM (Fernández-Real et al., 2011; Zhang et al., 2017; Al Dubayee et al., 2018).

Therefore, this research aimed to evaluate the effect of *T. diversifolia* extract (TDE) on leptin, adiponectin, and IR concentrations in rats with streptozotocin (STZ)-induced diabetes.

MATERIALS AND METHODS

Study design

The approval (number: 2.KE.091.05.2018) for this study was obtained from the Animal Care and Use Committee, Faculty of Veterinary, Universitas Airlangga, Surabaya, Indonesia. *T. diversifolia* was obtained from a traditional flower market in Magelang, Central Java, Indonesia, and the leaves were extracted by maceration with 70% ethanol (Wahyuningsih et al., 2019). The extract was diluted in 0.25% sodium carboxymethyl cellulose (CMC-Na) suspensions.

Research procedures

***Experimental animals*:** Male Wistar rats aged 2 to 3 months and weighing 150 to 200 g (*Rattus norvegicus*) obtained from the animal laboratory, Universitas Airlangga, were used in this study. The rats were adapted to laboratory conditions, which included food pellets and tap water at room temperature for 7 days.

Next, the rats were divided into four groups, with six animals per group. The control group (D0) received normal saline, and the positive group (D1) was administered 0.25% CMC-Na. One treatment group (D2) received TDE orally at 100 mg/kg body weight (bw), and the comparison group (D3) received catechin at 10 mg/kg bw for 7 days. Groups D1, D2, and D3 were induced with a single intraperitoneal dose of STZ (Sigma-Aldrich Co., St. Louis, MO, USA) at 100 mg/kg bw. The rats were sacrificed to collect blood and tissue samples on day 8. The kidney and pancreas were immersed in 10% buffered neutral formalin for immunohistochemistry and hematoxylin-eosin staining (Al Drees et al., 2017).

***Sample collection and preparation*:** BG and total serum cholesterol concentrations were measured using the colorimetric method (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). First, 10 µL of serum was incubated with 1 mL of glucose reagent or 1 mL of cholesterol reagent for 10 min. Next, the absorbance of the colored solution was measured at 546 nm. Then, the BG and serum cholesterol concentrations were determined by comparing the absorbance values of the samples with those of the standard solutions (Varshney et al., 2020). In addition, the concentrations of leptin and adiponectin were measured using the quantitative sandwich enzyme-linked immunosorbent assay technique (Elabscience, Wuhan, China). First, a microplate was precoated with the rat monoclonal antibody for leptin and adiponectin. Next, the sample was added to the microplate. After incubation and washing, an enzyme-linked detection antibody for

Table 1. Average concentrations of blood glucose, total serum cholesterol, leptin, and adiponectin in the groups (unit: mg/dL)

Group	Blood glucose	Total serum cholesterol	Leptin	Adiponectin
D0	138.50±81.83[a]	121.50±11.86[a]	1.98±0.12[a]	38.99±1.86[b]
D1	403.83±160.75[c]	161.33±14.88[b]	3.73±0.75[c]	31.81±2.56[a]
D2	209.16±33.17[b]	118.16±5.56[a]	2.65±0.92[b]	40.49±1.20[c]
D3	190.83±77.83[b]	110.16±2.92[a]	2.71±0.58[b]	37.52±0.77[b]

Data are expressed as mean±SD (n=6).
Different letters (a-c) indicate a significant difference ($P \leq 0.05$) in the same column.
D0, control group; D1, positive group; D2, treatment group; D3, catechin group.

leptin and adiponectin was added. Then, a substrate for the enzyme attached to the detection antibody was added. Lastly, the color of the reaction mixture was measured at 450 nm (Samarghandian et al., 2017).

The concentrations of kidney IR and pancreatic macrophage CD14 were determined using indirect immune peroxidase monoclonal antibodies and secondary anti-peroxidase antibodies. They were stained with diaminobenzidine (Bioss Antibodies, Woburn, MA, USA), labeled with a streptavidin-biotin and horseradish peroxidase, and analyzed using Starr Trek Universal detection system (ScyTek Laboratories, Inc., Logan, UT, USA) (Zhang et al., 2017).

Statistical analysis

The parametric data were compared among the groups using one-way analysis of variance. In the presence of a significant difference, analysis was performed using a Duncan's post hoc test with a 95% confidence level (α= 0.05). Nonparametric data from the immunostaining and histopathological experiments were scored and analyzed using the Kruskal-Wallis and Mann-Whitney U tests; statistical significance was defined as $P \leq 0.05$ for all analyses.

The areas containing immune reactive islets of Langerhans cells and in the epithelia of the tubules and kidney mesangial cells in pancreatic and kidney tissue sections, respectively, were examined using an Olympus light microscope (Olympus Life Science, Waltham, MA, USA) at 400× magnification. Conversely, the renal IR and CD14 macrophage concentrations were determined based on the presence of brownish aggregates (positive reaction) or the absence of brown aggregates (negative reaction), color intensity, and the area of positive reaction. The immunoreactions were scored 0 (no alteration), 1 (1~30% positive area), 2 (31~50% positive area), or 3 (51~100% positive area) (Sahrial and Solfaine, 2019).

RESULTS AND DISCUSSION

The typical symptoms of T2DM include high BG concentrations, which are caused by peripheral insulin resistance and impaired insulin secretion. Therefore, the standard test for determining the metabolic status of a patient or animal with diabetes mellitus is measurement of the BG and insulin concentrations.

The concentrations of BG, total serum cholesterol, and leptin were significantly decreased in the D2 and D3 groups ($P \leq 0.05$) compared with those in the D1 group (Table 1). The concentration of adiponectin was significantly increased in the D2 and D3 groups ($P \leq 0.05$) compared with that in the D1 group. Meanwhile, the D3 group had the lowest average BG concentration. There was a significant difference ($P \leq 0.05$) in the average BG concentrations among all the groups and between groups D0 and D1, groups D1 and D2, and groups D1 and D3.

On the other hand, group D2 had the highest average adiponectin concentration, and group D1 had the highest average leptin concentration. There was a significant difference ($P \leq 0.05$) in the average leptin concentration between groups D0 and D1, D1 and D2, and D1 and D3 (Fig. 1).

There was a significant difference in the average adiponectin concentration between groups D0 and D1, D1 and D2, D1 and D3, and D2 and D3. The histopathology scores for necrotic and infiltrated cells in group D1 were significantly greater than those in group D2 ($P \leq 0.05$).

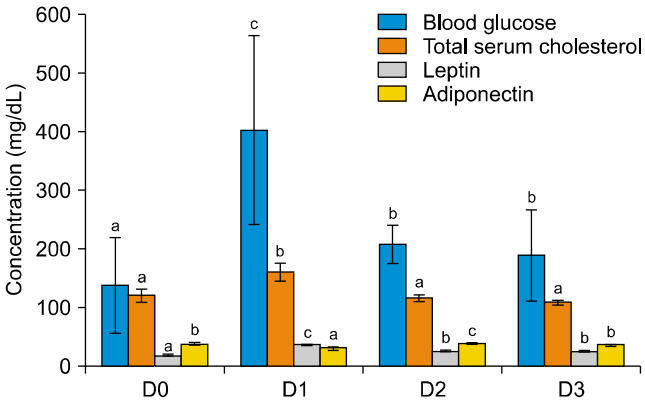

Fig. 1. Concentrations of blood glucose, total cholesterol, leptin, and adiponectin in the groups. Data are expressed as mean±standard deviation. Different letters (a-c) indicate a significant difference ($P \leq 0.05$). D0, control group; D1, positive group; D2, treatment group; D3, catechin group.

Table 2. The histopathological and immunoreaction scores of insulin receptor and macrophage CD14 in the groups

Group	Insulin receptor	Macrophage CD14	Necrotic cells	Infiltrated cells
D0	8.67±1.96[c]	1.67±0.81[a]	0.12±0.10[a]	0.03±0.08[a]
D1	3.88±1.16[a]	9.83±1.72[c]	3.06±0.37[c]	0.73±0.20[c]
D2	6.33±1.50[b]	7.00±1.54[b]	1.10±0.27[b]	0.13±0.10[a]
D3	6.17±1.60[b]	6.33±2.25[b]	1.13±0.30[b]	0.26±0.34[b]

Data are expressed as mean±SD (n=6).
Different letters (a-c) indicate a significant difference ($P \leq 0.053$) in the same column.
D0, control group; D1, positive group; D2, treatment group; D3, catechin group.

Moreover, the immune positive scores for IR and CD14 macrophage concentrations were significantly different between the treatment and positive groups ($P \leq 0.05$; Table 2).

The cells in the islets of Langerhans in group D1 had severe inflammation and necrosis (Fig. 2). Meanwhile, the immunohistochemistry scores indicated a significant decrease in IR concentrations in group D1 compared with that in group D2 ($P \leq 0.05$; Fig. 2).

Previous studies showed that STZ elevates BG concentrations (Samarghandian et al., 2017). Hyperglycemia can be observed in diabetic rats because of the destruction of pancreatic islet cells; some cells are normal and can secrete insulin in STZ-induced diabetic rats (Balbaa et al., 2016). Meanwhile in other type 2 diabetic rats, during insulin resistance, these insulin hormones could not work normally even with large amounts of insulin secreted by a patient with diabetes. *T. diversifolia* leaf extract improves insulin action at the cellular level. For example, Olukunle et al. (2014) reported that *T. diversifolia* leaf extract attenuated glucose concentrations in rats with diabetes.

Our results showed that the rats treated with TDE and catechin had lower glucose concentrations than those in the D1 group, although treatment with TDE significantly reduced the BG concentration. However, Thongsom et al. (2013) observed that the total phenolic content and antioxidant capacity of *T. diversifolia* leaves were 55.92±4.45 mg/g gallic acid equivalents and 93.1±37.9 μM Trolox equivalent antioxidant capacity/mg dry weight, respectively. Phenol has therapeutic properties for different diseases because it has anti-inflammatory and antioxidant activities (Šibul et al., 2016). *T. diversifolia* leaves, stems,

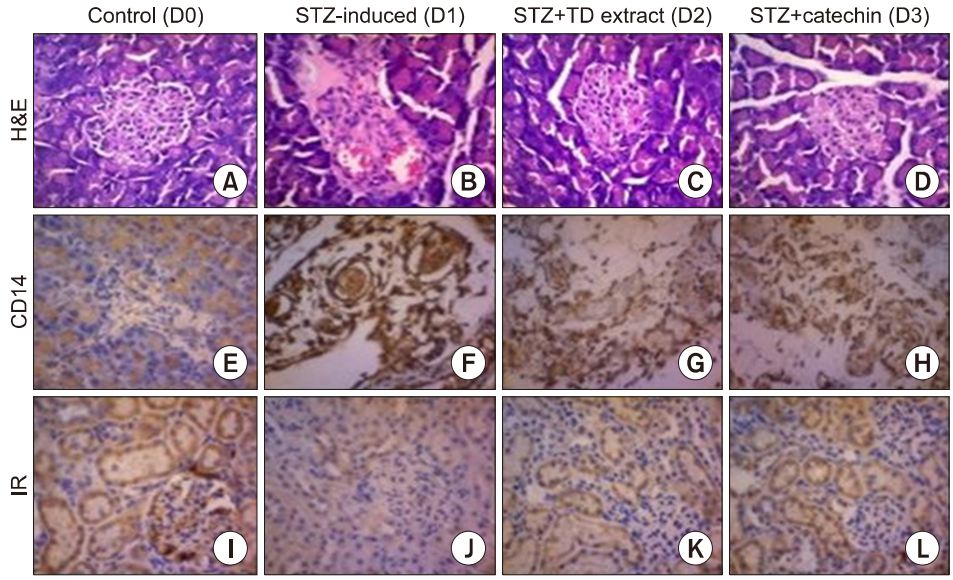

Fig. 2. Sections of the islet of Langerhans in the experimental groups. Morphological changes of vacuole degeneration, necrotic lesions, and interstitial inflammation of the mononuclear area of pancreatic β-cells in control rats (A), streptozotocin (STZ)-induced rats (B), STZ-induced rats treated with *Tithonia diversifolia* extract (C), and STZ-induced rats treated with catechin (D). The samples (A-D) were stained with hematoxylin-eosin and observed at 400× magnification. The CD14-positive cells were expressed along with the interstitial fat tissue of the pancreas, indicating activated macrophages. Increased numbers of CD14-positive cells in the affected periphery of the islet of Langerhans in control rats (E), STZ-induced rats (F), STZ-induced rats treated with *T. diversifolia* extract (G), and STZ-induced rats treated with catechin (H). The cells (E-H) were immunohistochemically stained, counterstained with hematoxylin, and viewed at 400× magnification. Distribution of renal insulin receptor (IR)-positive in the area of mesangial cells and surrounding epithelial tubules in control rats (I), STZ-induced rats (J), STZ-induced rats treated with *T. diversifolia* extract (K), and STZ-induced rats treated with catechin (L). The cells (I-L) were immunohistochemically stained, counterstained with hematoxylin, and viewed at 400× magnification. D0, control group; D1, positive group; D2, treatment group; D3, catechin group; TD, *T. diversifolia*.

and roots contain alkaloids, tannins, flavonoids, saponins, and terpenoids (Mabou Tagne et al., 2018). Flavonoids have antidiabetic properties, reduce oxidative stress (Testa et al., 2016), and exert a modulatory effect on glucose transporters (GLUTs) by enhancing the expression of *GLUT-2* gene in β cells and GLUT-4 translocation (Hajiaghaalipour et al., 2015). Therefore, according to Vinayagam and Xu (2015), food-derived flavonoids improve glucose metabolism. Meanwhile, saponins serve as antidiabetic agents by regulating BG concentrations and preventing complications due to their antioxidant capacity (Elekofehinti, 2015). Choi et al. (2017) reported that saponin helped manage diabetes mellitus in patients with chronic alcohol consumption. Patel et al. (2012) found that saponin significantly decreased BG concentrations and improved plasma insulin.

Catechin, a substance commonly found in green tea, has antidiabetic effects; it can elevate insulin concentration and block sodium-dependent GLUT-1 formation (Park et al., 2014). In rats induced with STZ, catechin was shown to prevent the activation of pancreatic islet cells (Kim et al., 2003; Song et al., 2003). However, in normal conditions, insulin functions as a hormone signaling cell receptor when glucose molecules enter from the blood circulation. Liu et al. (2013) reported that the consumption of green tea significantly reduced the fasting BG concentration. Another study reported that catechin and catechin-containing foods improved hyperglycemia and dyslipidemia in patients with T2DM (Nagao et al., 2009). Furthermore, the treatment with large amounts of catechin reduces plasma glucose levels more because catechin causes a remarkable increase in glucose oxidation in skeletal muscles (Alipour et al., 2018). Generally, glucose oxidation provides energy, and its rate depends on the entry rate of glucose into the cells. Therefore, elevating insulin concentrations does not reduce BG concentrations in patients with T2DM and insulin resistance.

Our results demonstrated a significant difference in the average leptin and adiponectin concentrations between the treatment and control groups. The leptin concentrations significantly decreased in the treatment group compared with those in the positive control group. Meanwhile, the treatment groups had higher adiponectin concentrations than the other groups, and the insulin concentration was significantly lower in the treatment group than in the control group.

Treatment with TDE increased the adiponectin and decreased leptin concentrations compared with those in the positive control group. The antioxidant components in *T. diversifolia* leaves protect against oxidative stress, which attenuates adiponectin concentrations. This study demonstrated improved insulin action and decreased leptin secretion in insulin-resistant rats induced by STZ after treatment with TDE.

In addition, the results showed that treatment with TDE decreased leptin concentrations in the positive control group. According to previous studies, *T. diversifolia* has phenolic components and antioxidant properties and can reduce cholesterol and low-density lipoprotein (LDL) concentrations (Geleijnse and Hollman, 2008; Olukunle et al., 2014). Furthermore, TDE was shown to improve the lipid profile, reduce body fat, and reduce total cholesterol concentrations in patients with T2DM (Nagao et al., 2009; Park et al., 2014; Samarghandian et al., 2017). Previous studies also revealed a positive relationship between the concentrations of leptin, triglyceride, LDL, BG, and hypertension in patients with T2DM (Uslu et al., 2012).

Leptin maintains body weight and regulates appetite, energy metabolism, islet cell growth in the pancreas, and insulin secretion. Therefore, it is a linking factor between obesity and an increased risk of cardiovascular disease. It also has a physiological function in preventing insulin resistance by regulating food intake or weight in patients with diabetes (Sarath and Rajkumar, 2011). Increased leptin concentrations in patients with obesity decrease pancreatic β-cell receptor response, thereby increasing insulin secretion. The resultant hyperinsulinemia causes an increase in leptin concentrations, leading to leptin and insulin resistance. Therefore, an increase in blood leptin concentrations is associated with an increase in body mass index and insulin resistance in patients with diabetes mellitus (Uslu et al., 2012). According to López-Jaramillo et al. (2014), an elevation in leptin concentration coincides with an increase in plasma insulin concentrations caused by leptin resistance.

Furthermore, adiponectin, a hormone derived from adipocytes and fat tissues and present in plasma, has antiatherogenic, antidiabetic, and anti-inflammatory functions and is correlated with severe obesity. Adiponectin concentrations are low in patients with obesity (Arita et al., 1999; Hecker et al., 2011; López-Jaramillo et al., 2014; Chakraborti, 2015). Meanwhile, adiponectin increases insulin sensitivity by reducing plasma fatty acid concentrations in patients with diabetes. It also improves fatty acid oxidation, which is mediated by AMP-activated protein kinase phosphorylation. This mechanism tends to increase glucose uptake and reduce glucose production in the liver; therefore, adiponectin deficiency leads to hyperglycemia, hyperinsulinemia, and insulin resistance (Arita et al., 1999; Hadžović-Džuvo et al., 2014; Liu et al., 2014).

We used an IR monoclonal antibody to detect the presence and distribution of renal IR in the epithelial cells of renal tubules and mesangial cells. The IR concentrations demonstrated the regulation of BG concentrations and an increase in the treatment and catechin groups. Therefore, the proliferation of IR-positive cells is likely to increase cellular glucose uptake. However, compared with the

treatment group (D2), the distribution of renal IR-positive cells was limited to the affected tubule epithelial and mesangial cells in the positive control group (D1). These data suggested differences in the distribution of IR-positive cells in interstitial tubules in the TDE-treated groups. An increased number of renal IR-positive cells was consistently observed in the STZ-induced rats in the D2 and D3 groups, following the changes in adiponectin concentrations.

Pancreatic histopathological results revealed a loss of islet of Langerhans structure. This finding indicated severe tissue destruction due to cellular inflammation and necrosis in the islet area, with the formation of vacuoles and loss of numerous β cells. The immunostaining results showed that the number of CD14-positive macrophages increased in group D1. However, CD14-positive cells are likely to have activated inflammatory signaling pathways in STZ-induced rats.

TDE significantly restored BG concentrations to normal in rats with STZ-induced diabetes in the treatment group and significantly reduced leptin concentrations more than catechin concentrations. Furthermore, the *T. diversifolia* leaf extract significantly elevated adiponectin levels in the treatment group compares with that in control group (D0 and D1).

In summary, this study demonstrated that *T. diversifolia* leaf extract decreases leptin and BG concentrations by increasing IR expression and reduces pancreatic tissue necrosis by suppressing macrophage CD14 concentrations in rats with STZ-induced diabetes.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: LM, RS. Analysis and interpretation: LM, RS. Data collection: LM, M. Writing the article: LM, RS. Critical revision of the article: RS, M. Final approval of the article: all authors. Statistical analysis: M, LM. Obtained funding: LM, M. Overall responsibility: all authors.

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