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RESEARCH ARTICLE

Antidiabetic effects of *Salvia triloba* and *Thymus praecox* subsp. *skorpilii* var. *skorpilii* in a rat model of streptozotocin/nicotinamide-induced diabetes

Muhammet Emin ÇAM, Sıla YILDIZ, Büşra ERTAŞ, Ayşe Eda ACAR, Turgut TAŞKIN, Levent KABASAKAL

ABSTRACT

Some *Salvia* and *Thymus* species of Lamiaceae family come into prominence with strong antidiabetic effects. Compared to the other species, there are limited studies on antidiabetic activity of *Salvia triloba* (ST) and *Thymus praecox* subsp. *skorpilii* var. *skorpilii* (TPS). The aim of this study was to adjust the dosage and to determine the antidiabetic effects of methanol extracts of ST and TPS in streptozotocin/nicotinamide-induced diabetic rats. Type II diabetes mellitus (T2DM) was induced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ) dissolved in 0.1 M cold citrate buffer (pH 4.5) at a dose of 55 mg/kg/body weight (b.w.) and nicotinamide (100 mg/kg/b.w.) was given prior to STZ injection. For adjusting dosage, oral glucose tolerance test (OGTT) was used while insulin tolerance test (ITT), OGTT, blood glucose levels and animal weights were used to evaluate the antidiabetic effects of ST and TPS. According to the OGTT, the most effective doses for ST and TPS were 200 mg/kg and 100 mg/kg, respectively. At the end of three weeks, blood glucose levels of control goup reached to 462.50 mg/dl, whereas ST and TPS-treated groups blood glucose levels decreased less than 200.00 mg/dl. In conclusion, the present study suggests that both of ST and TPS methanolic extracts may be of therapeutic benefit in diabetes and thus need to further studies.

Keywords: Type II diabetes mellitus ; *Salvia triloba* ; *Thymus praecox* ; Streptozotocin-Nicotinamide ; herbal medicines ; antidiabetic

1. Introduction

The genus *Thymus* belongs to the family Lamiaceae, is represented in Turkey with 38 species, and the ratio of endemism in the genus is 53% [1].

The dried herbal parts of several *Thymus* species are used in herbal tea, condiment and folk medicine. Studies demonstrated that the *Thymus* species could have antiinflammatory, antifungal, antiviral, antioxidant, anticancer and antidiabetic effects [2].

The essential oils of some *Thymus* species are characterized by the presence of high concentration of the isomeric phenolic monoterpenes thymol and/or carvacrol [1, 2].

The essential oil of *Thymus praecox* subsp. *skorpilii* var. *skorpilii* (TPS) contains α-pinene, camphene, β-myrcen, *o*-cymene, limonene, eucalyptol, terpinene, thymol, carvacrol, caryophyllene [3].

The genus *Salvia* L. (Lamiaceae) includes around 1000 species that have almost cosmopolitan distribution. The

Muhammet Emin Çam, Sıla Yıldız, Büşra Ertaş, Ayşe Eda Acar, Levent Kabasakal
Department of Pharmacology, Faculty of Pharmacy, Marmara University, Haydarpaşa 34668 İstanbul, Turkey

Turgut Taşkın
Department of Pharmacognosy, Faculty of Pharmacy, Marmara University, Haydarpaşa 34668 İstanbul, Turkey

Corresponding Author:
Muhammet Emin Çam
e-mail: muhammet.cam@marmara.edu.tr

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some species of *Salvia* is frequently used as herbal tea and source of essential oils.

Salvia triloba L. (ST) (Syn. *S. fruticosa* Mill.) leaves have been used for treatment of various skin, blood, and infectious ailments as well as ailments of the digestive, circulatory and respiratory systems. ST is growing in the Southwestern Anatolia, especially in Muğla. It is locally known as "Anadolu Adaçayı" [3].

The essential oil of *S. triloba* (ST) contains α-pinene, camphene, β-pinene, myrcene, 1,8-cineole, γ-terpinene, *cis*-thujone, *trans*-thujone, camphor, terpinene-4-ol, *trans-(E)*-caryophyllene, aromadendrene and α-humulene [4].

TPS is a traditional herbal medicine has attracted considerable interest to treat type II diabetes mellitus (T2DM) in Merzifon region of Turkey [5]. There have been a limited number of studies examining the effects of TPS. Studies showed that *Salvia* species have antidiabetic effects but there have not been any study on antidiabetic effect of ST. Therefore, this study was designed to investigate the hypoglycemic properties and the doses of methanol extracts of *Thymus praecox* subsp. *skorpilii* var. *skorpilii* and *Salvia triloba*.

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia resulting from impaired insulin secretion, insulin resistance, or both and also it is a chronic, complex disease that requires continuous medical care along with multifactorial risk reduction strategies other than glycemic control [6]. According to the International Diabetes Federation (IDF), DM is 8th most important cause of death and 382 million people living with diabetes throughout the world in 2013 (approximately 7 million in Turkey) and over 316 million of diabetic people are under high risk because of impaired glucose tolerance [7]. World Health Organization (WHO) demonstrated that 422 million people have diabetes in 2014. The global prevalence of diabetes among adults (>18) is 8.5%. WHO projects that DM will be 7th leading cause of death in 2030. On the other hand, IDF estimates that the number of people with diabetes will rise to 592 million in 2035.

T2DM is the most common form of DM and comprises 90% of diabetic people. T2DM are known as adult-onset or non-insulin-dependent, primarily seen in middle-aged adults (>40), in despite of type 1 DM (T1DM) which is typically diagnosed at a much earlier stage. However, T2DM have become more common in young adults, teens and children in recent years. In T2DM, the pancreas initially produces insulin, but does not produce enough insulin or the cells of the body are unable to use insulin by the reason of post-receptor disorders. T2DM is considerably the result of excess body weight and physical inactivity [8].

Symptoms may be similar to T1DM, but generally people are unaware of its. Consequently, the disease may be diagnosed several years after onset. In contrast to T1DM, most of people with T2DM do not have to take insulin during their lifetime, they need to be physically active, maintain healthy body weight and eat healthy diets [9].

The aim of this study is to support the medicinal treatment of T2DM and to reduce dose or to prevent possible side effect of likely used oral antidiabetics by the usage of extracts.

2. Results

2.1. Hypoglycemic Effects In Normal Rats

To identify potential antidiabetic effects and the most effective doses of TPS methanolic extract (TPSE) and ST methanolic extract (STE), OGTT was addressed in normal rats. After 12 h of fasting, treatments were carried out and 30 min later a single dose of glucose (4 g/kg b.w.) was applied. As shown Table 1, blood glucose levels (BGL) in all groups showed a clear increase in one hour following oral glucose challenge (at 30 and 60 min), verifying the induction of hyperglycemia. BGL in control group is in tendency to return to normal levels at 120 min similarly to other five groups treated with 100, 200 mg/kg (b.w.) TPSE and 100, 200, 400 mg/kg (b.w.) STE. STE at 200 mg/kg have shown apparent significant reduction in blood glucose levels ($p<0.001$) in one hour compared to control. On the contrary, 100 and 400 mg/kg STE given rats did not reduce BGL when compared to the control. Therefore, 200 mg/kg was chosen for STE group in the experiment.

On the other hand, TPSE treated groups indicated similar BGL lowering effect but at the first hour of the test, 100 mg/kg TPSE group's BGL lowering effect was slightly stronger ($p<0.005$). Since the lower dose would have fewer side effects, 100 mg/kg was chosen for TPSE.

Table 1. Blood glucose levels obtained by oral glucose tolerance test in normoglycemic rats for dose determination.

	Blood glucose levels (mg/dl)				
Groups	**0 min**	**30 min**	**60 min**	**120 min**	**180 min**
Control	88±6.96	193±12.08	182±8.16	100±9.70	93±2.77
STE-100	90±4.80	175±10.59*	159±5.66	91±4.07	85+2.99
STE-200	88±5.40	161±14.12***	135±10.02***	89±6.50	89±3.52
STE-400	91±4.51	167±12.28	160±6.94	110±3.80	96±2.49
TPSE-100	100±1.91	145±9.28	142±7.21*	104±5.62	93±3.40
TPSE-200	93±2.30	154±2.95	147±3.94	104±3.75	93±2.34

Values are expressed as Mean ± SEM (Standard error mean); Values are calculated using two-way ANOVA followed by Bonferroni post-tests; ***p<0.001 and *p<0.05 compared to control group; n=6.

2.2. Effects of TPSE and STE on Body Weight Change

Animals of the same weight range were used in experimental protocol. The body weights of experimental animals are shown in Figure 1. Oral administration of TPSE and metformin considerably maintain the weights in three weeks period compared to diabetes group. However, STE group started losing weight (p<0.05) beginning from second week and continued in third week (p<0.05) compared to control group. Last week, all groups lost weight in a small amount. The body weights of non-treated diabetic rats decreased almost 11%.

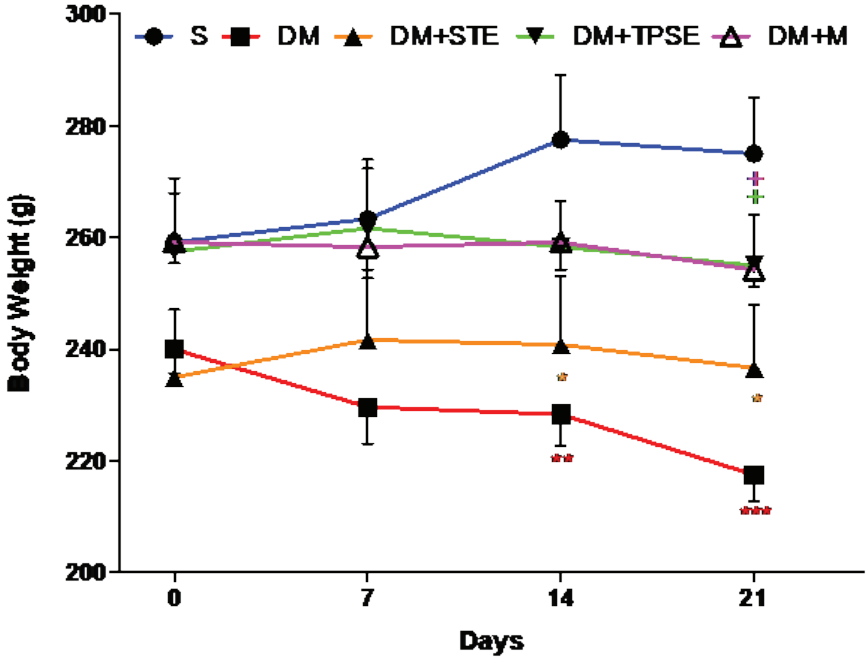

Figure 1. Effects of STE and TPSE treatment on body weight in diabetic rats for 21 days. Each group (n=6) represents Mean ± SEM. Data was analyzed by using two-way ANOVA followed by Bonferroni posttests; ***p<0.001, **p<0.01 and *p<0.05 compared to control group; +p<0.05 compared to diabetes group.

2.3. Effects of TPSE and STE on Blood Glucose Levels

Figure 2 demonstrates the alterations in BGL of experimental groups. Non-treated diabetic rats showed a significant increase ($p<0.0001$) in BGL compared with the other groups and their BGL increased gradually during the three weeks. Blood glucose levels of TPSE and STE treated rats lowered considerably ($p<0.0001$ and $p<0.01$, respectively) in first week compared to the those of beginning and these decreases lasted for three weeks. At the end of three weeks, the lowest average blood glucose level in treatment groups belongs to Metformin group (132.50±7.40 mg/dl). However, BGL reduction ratio of TPSE and STE groups are similar to Metformin group. The percentage reduction in blood glucose levels after 3 weeks in TPSE and STE groups are 58 and 45, respectively. After three weeks of TPSE treated group, BGL reduced to 147.33±9.83 mg/dl, which was only 11% higher than metformin treated group.

2.4. Effects of TPSE and STE on Oral Glucose Tolerance Test (OGTT)

After 12 h of fasting, treatments were carried out in all groups and a single dose of glucose (2 g/kg b.w.) was applied 30 min later. The levels of blood glucose obtained 30, 60, 120 and 180 min after glucose intake. Table 2 illustrates changes in BGL during OGTT. All groups showed significant augmentation ($p<0.0001$) in BGL during one hour following oral glucose challenge, verifying the induction of hyperglycemia.

As expected, metformin treated group significantly lowered BGL beginning from 30 min compared to diabetes ($p<0.0001$) and treatment groups ($p<0.001$). BGL increases in TPSE and STE groups are 2.39 and 2.12-fold in first hour, respectively. However this increase in Metformin group is just 1.27-fold. It demonstrates that Metformin protects BGL stronger than treatment groups and tolerate it faster. However, the BGL lowering effect between TPSE and metformin was not considerably different at the second hour. TPSE was able to reduce BGL similarly to metformin.

TPSE and STE demonstrated an important blood glucose reduction (60% and 59%, respectively, $p<0.001$) at the second hour compared to the first hour.

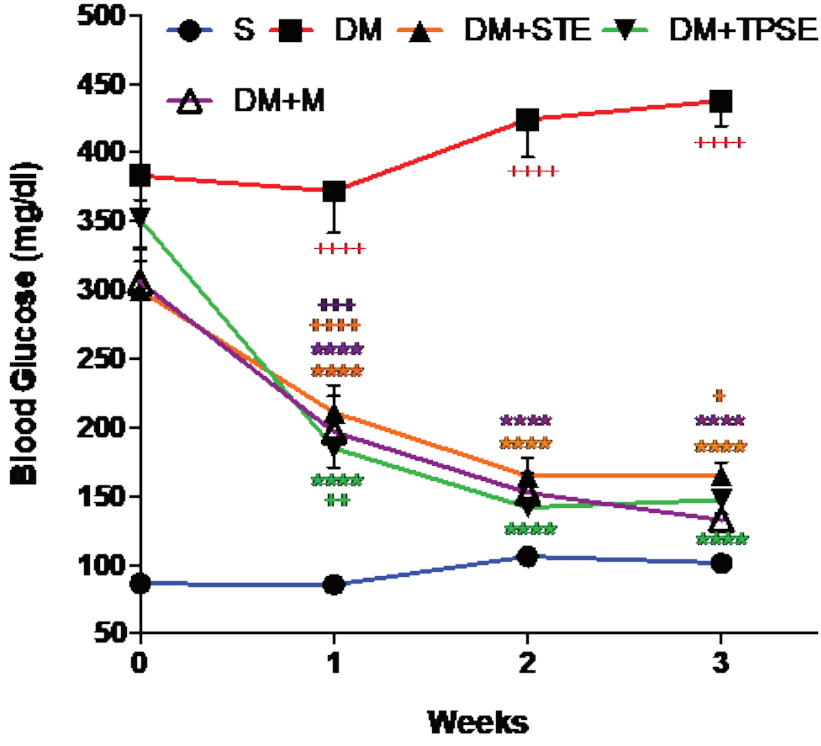

Figure 2. Changes in the level of blood glucose after the administration of ST and TPS treatments for 3 weeks. Each group (n=6) represents Mean ± SEM. Data was analyzed by using two-way ANOVA followed by Bonferroni posttests; ****$p<0.0001$ compared to diabetes group; $^{++++}p<0.0001$, $^{+++}p<0.001$, $^{++}p<0.01$, $^{+}p<0.05$ compared to control group.

Table 2. The effects of TPSE and STE on Oral Glucose Tolerance Test in diabetic rats

Blood glucose levels (mg/dl)					
Groups	**0 min**	**30 min**	**60 min**	**120 min**	**180 min**
Control	86±2.95	146±8.86	123±5.13	90±2.76	85±3.73
DM	135±23.08	347±60.97^{a4}	317±52.89^{a4}	225±50.13^{a3e2}	183+47.39^{a1f4g3}
DM+STE	120±7.86	285±9.94^{a3}	254±21.95^{a2}	105±7.15^{b2e4g3}	96±3.98^{f4h4}
DM+TPSE	98±3.33	288±19.96^{a3}	235±19.18^{a2}	94±8.58^{b2e4g3}	95±2.83^{f4h3}
DM+M	101±4.99	145±13.90^{b4c3d3}	128±10.61^{b4c2d1}	80±4.75^{b3}	86±4.17^{b4}

Values are mean±SEM. n=6, [a]compared to control; [b]compared to diabetes; [c]compared to STE; [d]compared to TPSE; [e]compared to 30 min; [f]compared to 30 min; [g]compared to 60 min; [h]compared to 60 min. [1]p <0.05; [2]p<0.01; [3]p<0.001; [4]p<0.0001.

2.5. Effects of TPSE and STE on Insulin Sensitivity

To evaluate insulin sensitivity, after 12 h of fasting, 1 U/kg (i.p.) insulin was applied and blood glucose levels were measured just before injection and 15, 30, 45, 60 and 90 minutes after the injection. The treatment groups showed significant impairment in insulin tolerance compared with diabetes group and diabetic rats showed remarkable reduction in BGL (Figure 3).

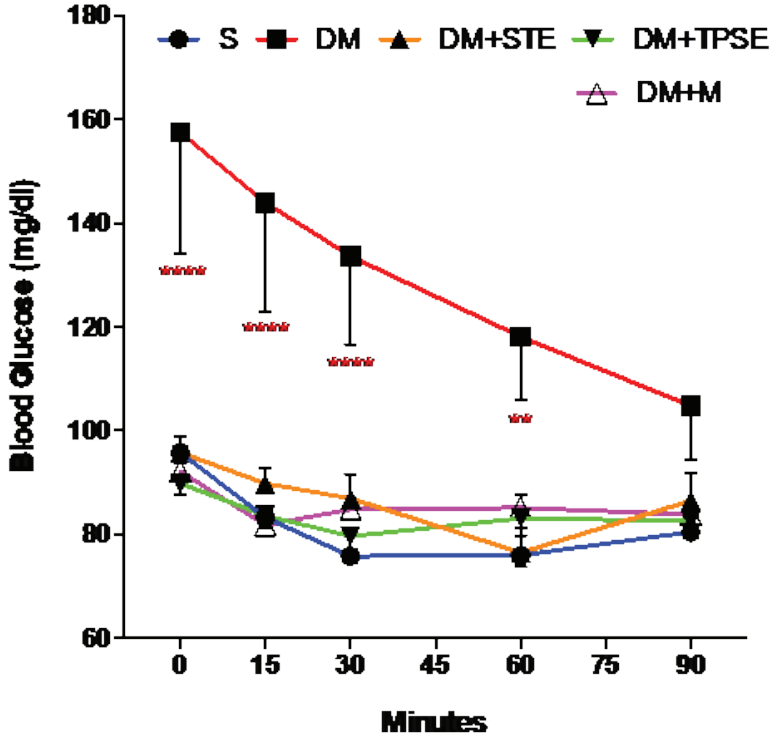

Figure 3. Changes in the level of blood glucose after insulin injection. Each group (n=6) represents Mean ± SEM. Data was analyzed by using two-way ANOVA followed by Bonferroni posttests; ****p<0.0001, **p<0.01 compared to control group.

3. Discussion

Type II diabetes mellitus is a chronic metabolic disease associated with genetic or lifestyle, is characterized by an increase in blood glucose. Recent studies has estimated that approximately 30% of patients with DM take complementary and alternative medicine [10]. Medicinal plants and its products proceed to be an important therapeutic alternative for relieving the ailments. Although new agents are developing, adverse effects are still a threat for the treatment of DM. For this reason, there is an increasing demand for agents known to have fewer side effects than oral hypoglycemic agents currently used in the medical care system. It is stated that antihyperglycemic natural products have fewer side effects than synthetic oral hypoglycemic agents. Since diabetes is a chronic disease, inadequate control of glucose levels would causes a number of complications like neuropathy, nephropathy and retinopathy. Thus, the research on plants for advancing the management of DM is a natural key.

STZ/NA induced T2DM in adult rats has been defined one of the most appropriate experimental models to evaluate the possible antidiabetic activity of medicinal plants [11]. The rats administered nicotinamide (100 mg/kg, i.p.) 15 min before STZ (55 mg/kg, i.p.) was found to develop moderate and stable non-fasting hyperglycemia. STZ has potent alkylating property and is particularly cytotoxic to the pancreatic beta cells, causes the death of pancreatic β-cell by alkylation of DNA and subsequent activation of poly (ADP-ribose) synthetase, resulting in formation of superoxide radicals [12]. As a result, STZ attenuates insulin release and synthesis. On the contrary, NA is an antioxidant agent that reveals protective effects against STZ by scavenging free radicals and produces T2DM [13].

The rats whose blood glucose levels between 200-400 mg/dl three days after induction were considered T2DM. In particular, the rats have over 400 mg/dl BGL were considered severely diabetic [14]. Therefore, animals having blood glucose more than 400 mg/dl were discarded from the study.

ST and TPS were used to lower blood glucose as a traditional medicine, in the present study we hypothesized to show the antidiabetic effect of their extract. It was observed that STE performed its action in a non-dose dependent manner; higher dose demonstrated less effect. 200 mg/kg STE caused significant hypoglycaemia and lowered BGL faster ($p<0.001$) compared to the other doses. On the other hand, TPSE treated groups indicated BGL lowering effect at the first hour of the test, 100 mg/kg TPSE group's BGL lowering effect was slightly stronger ($p<0.05$).

Administration of TPSE and STE for 21 days considerably reduced BGL as compared to diabetic rats. Like previous studies in DM, STZ/NA induced diabetic rats demonstrated significant increase in blood glucose level compared to the control group [15]. The methanolic extracts of the plants decreased BGL in STZ/NA induced diabetic rats. However, TPSE is more effective ($p<0.0001$) in the reduction of BGL by 47% in first week, at the same time STE and metformin started lowering blood glucose level less severe by 30% and 36%, respectively compared to TPSE. After 3 weeks in TPSE and STE groups, BGL were in normal ranges.

As usual, all rats started losing weight from the induction of DM to beginning of treatment after three days except of control group. At the same time, losing weight in this period shows that diabetes has been successfully induced. Induction of DM is associated with the characteristic loss of body weight in diabetic rats [16], as a result of muscle wasting and catabolism of tissue proteins. Oral administration of TPSE and STE helped rats to protect their body weight in the first two weeks compared to diabetic group which might be the reason of its protective effect in regulating muscle losing, as a consequence of reversal of gluconeogenesis, might also be due to the enhancement in insulin secretion and glycemic control. However, in third week, all groups lost weight in a small amount, it is possible that to fast animals before OGTT and ITT caused this small losing. In three-week period, non-treated diabetes group lost maximum weight by 16%. The rats in TPSE and STE treated groups lost weight by 7% and 8%, respectively.

A key phase of metabolic syndrome is an insulin resistance state, forming the most important risk factor for the progress of glucose intolerance and DM [17]. For this reason, interferences to diminish insulin resistance may delay the progress of DM and its complications. OGTT is one of the most important parameters for evaluating the influence of hypoglycemic medicines [18]. In OGTT, BGL enhanced in time and was sustained until 2 hours in diabetic rats. Whereas, TPSE and STE significantly lowered BGL in diabetic rats and therefore the extracts might augment glucose utilization by peripheral tissues and enhancing the glycogen depots in the liver due to repairment of postponed insulin response. In glucose-fed diabetic rats, the raised BGL remained higher after 180 min (182.67±47.39 mg/dl). It was 35% higher than beginning. At the same time, TPSE and STE considerably inhibited raises in BGL during OGTT in STZ/NA diabetic. In metformin-treated group, BGL increase was 52% in 30 min

and only 18% in 60 min compared to the those of beginning. These values for TPSE and STE groups were 178%-127% and 137%-113%, respectively. Briefly, metformin-treated group quickly balanced BGL such as control group compared to other treatment groups. Consequently, all treatment groups significantly enhanced glucose tolerance and the possible effect was created by plasma insulin. Plasma insulin rising was provided by the pancreatic secretion of insulin from current β-cells or its release from bound insulin [19].

According to the data obtained by tests, TPSE and STE lowered blood glucose level, enhanced glucose tolerance and prevented hyperinsulinemia in diabetic rats. The results indicated that plant extracts used in therapy can improve insulin sensitivity.

TPSE also regulated the hyperglycemic state in OGTT, which was similar with the baseline within 120 min as well as standard drug. Furthermore, the fact that STE showed weaker effects does not mean that it is a weak substance. The certain mechanism for antidiabetic activity of TPSE and STE will require further detailed study.

4. Conclusion

This study indicated first time the antidiabetic effect of methanolic extracts of *Salvia triloba* and *Thymus praecox* subsp. *skorpilii* var. *skorpilii*. TPSE showed stronger antidiabetic effects compared to STE group. It is exciting to observe that TPSE lowered hyperglycemia level in STZ/NA-induced rats that was similar to the standard metformin. However, STE showed similar effects to TPSE in some tests indicating that STE also has potent antidiabetic effects. Thus, the outcomes from the more comprehensive, detailed researches and clinical trials based on these two potential plants may lead to develope a new alternative therapeutic agent in the treatment of T2DM.

5. Materials and Methods

5.1. Collection and Identification of Plant Material

Thymus praecox subsp. *skorpilii* var. *skorpilii* aerial parts were collected during the flowering stage from Bursa-Uludağ, Turkey. The taxonomic identity of the plant was confirmed by Dr. İsmail Şenkardeş. The voucher specimens were deposited in the herbarium of the Faculty of Pharmacy, Marmara University; herbarium numbers: MARE:18081. *Salvia triloba* L. (Syn. *Salvia fruticose* Mill.) aerial parts was purchased from Zeytinburnu Medical plants garden in Istanbul, Turkey.

5.2. Preparation of Extracts

The aerial parts of *Thymus praecox* subsp. *skorpilii* var. *skorpilii* were extracted using the maceration method with methanol solvent for 7 days. After complete extraction, the sample was filtered through filter paper and the solvent was evaporated using a rotary evaporator and the crude extract was kept in refrigerator at 4 °C for their future use.

Salvia triloba aerial parts were extracted in a Soxhlet extractor with methanol:water (4:1, v/v). The extract was filtered through filter paper and the solvent was evaporated using a rotary evaporator and the crude extract was kept in refrigerator at 4 °C for their future use.

5.3. Chemicals

Glucose, streptozotocin (STZ), nicotinamide (NA), metformin were purhased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Insulin was purchased from Eli Lilly, under the brand name Humulin®.

5.4. Animals

Female adult Sprague-Dawley (SD) rats (200-300g) were obtained from Experimental Animal Application and Research Center (DEHAMER) of Marmara University. The rats were kept at a constant temperature (22±2°C) with 50% humidity, 12-h light-dark cycles and were allowed *ad libitum* access to standard rat chow and water. The study was approved by the Marmara University, Animal Experiments Local Ethics Committee (MÜHDEK) (098.2016.mar).

5.5. Experimental Design

The dose selection for the diabetic study was guided by the results obtained from the oral glucose tolerance test in this study, which showed that the most effective dose was 100 mg/kg body weight (b.w.) for TPSE and 200 mg/kg b.w. for STE.

Diabetes was induced by intraperitoneal (i.p.) injection of STZ/NA. After an overnight fasting (16 h), all rats were given a single i.p. injection of NA (100 mg/kg/b.w. in normal saline) to minimize the pancreatic destruction. 15 minutes after injection of NA, to create type-2 diabetes, STZ (55 mg/kg/b.w. in 0.1 M cold citrate buffer, pH 4.5) was injected [20]. After 72 h, the blood glucose levels were monitored with a glucometer (Contour™ TS, Bayer Diagnostics) and the rats with plasma glucose levels >200 mg/dl were selected for

further study [21]. Treatments (TPSE, STE, metformin) were carried out after glucose measurement and lasted for 3 weeks except decapitation day.

Five groups, each containing 6 rats, were formed as follows:

- Diabetic group (DM); Vehicle-treated

- Diabetic + STE group (STE); Diabetic rats treated daily by gavage with 200 mg/kg STE

- Diabetic + TPSE group (TPSE); Diabetic rats treated daily by gavage with 100 mg/kg TPSE

- Diabetic + Metformin group (M); Diabetic rats treated daily by gavage with 400 mg/kg Metformin

- Healthy group (S); Vehicle-treated

The rats were weighed and blood samples were collected from the tail vein at the beginning and weekly until decapitation. On the 15th day, insulin tolerance test (ITT) and 18th day oral glucose tolerance test (OGTT) was applied to all rats after an overnight fasting. On day 21, the rats were decapitated. The timeline for the experimental design is shown in Figure 4.

5.6. Assessment of Insulin Sensitivity

Insulin tolerance test (ITT) was performed to evaluate insulin resistance in all rats [22]. After 12 h of fasting, animals received 1 UI/kg of insulin (i.p.) and blood glucose level was measured just before insulin injection and at 15, 30, 60, 90 min after injection from tail vein [23].

5.7. Oral Glucose Tolerance Test (OGTT)

An oral glucose tolerance test (OGTT) was applied on each rat to evaluate the ability to regulate glucose metabolism and to measure of the glucose induced insulin secretion and its mediated glycemic changes. After 12 h of fasting, treatments were carried out and a single dose of glucose (2g/kg, b.w.) was given by gavage 30 minutes later. Glucose concentrations were measured in the blood collected from the tail vein at 0 (just before glucose administration), 30, 60, 120, 180 min following the glucose injection [10].

5.8. Dose Determination

Female adult SD rats (200-300g) were divided into six groups (n=6). In 1th group, normal rats were treated by saline; in 2nd and 3rd group, TPSE was given in two different dose 100 and 200 mg/kg; in 4th, 5th and 6th group, STE was given in three different dose 100, 200 and 400 mg/kg, respectively. To determine the most effective dose for STE and TPSE, OGTT was applied in the same procedure but only glucose was given in a different dose 4 g/kg, b.w [24]. The dose was changed to observe the blood glucose changes more clearly.

5.9. Statistical Analysis

All data are expressed as mean ± SEM. Two-way ANOVA with Bonferroni post test was performed using GraphPad Prism version 6.00 for Mac OS X, GraphPad Software.

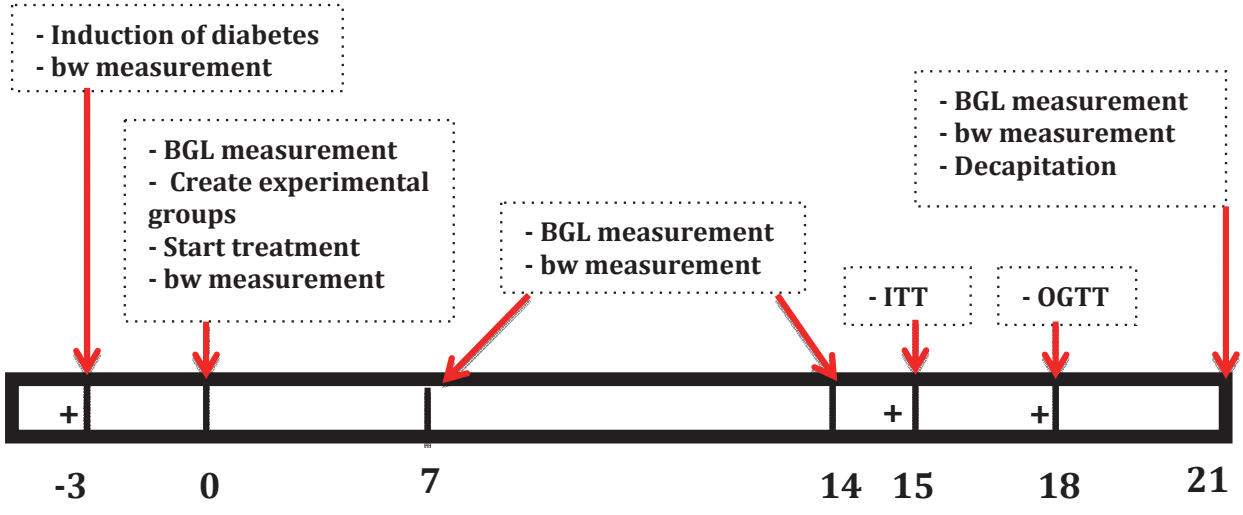

Figure 4. Schematic representation of the experimental design. "+" indicates 12-h fasting

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Abbreviations

ST: *Salvia triloba*

TPS: *Thymus praecox* subsp. *skorpilii* var. *skorpilii*

STZ: Streptozotocin

NA: Nicotinamide

OGTT: Oral glucose tolerance test

ITT: Insulin tolerance test

T1DM: Type I diabetes mellitus

T2DM: Type II diabetes mellitus

IDF: International Diabetes Federation

WHO: World Health Organization

SD: Sprague-Dawley

TPSE: *Thymus praecox* subsp. *skorpilii* var. *skorpilii* methanolic extract

STE: *Salvia triloba* methanolic extract

ANOVA: Analysis of Variance

SEM: Standard error of means

BGL: Blood glucose level

bw: Body weight

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