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Isatis Glauca Subsp. Sivasica Extract Contributes To The Diabetic Wound Healing Process By Increasing The Collagen And Nitric Oxide Content

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ABSTRACT

Isatis L. genus is highly endemic in Turkey and it is traditionally suggested for the wound management. In this study, we have reported the effect of the I. glauca subsp. sivasica extract on the diabetic wound healing process. A diabetic model was generated in Wistar rats using streptozotocin injection. The rats were divided into two main groups: the control group and the Isatis group. Full thickness excisional skin wounds were created by using a biopsy punch. The Isatis group was treated with single daily dose I. glauca subsp. sivasica extract (50 mg/kg). The rats were sacrificed on day 3 or day 7 after wounding. The dominant phenolic compounds identified with RP-HPLC-DAD from the Isatis extract were the benzoic acid and the vanillic acid. The *Isatis* extract significantly accelerated the wound healing process considering the wound closure rates (WCR). Moreover, the levels of collagen and nitric oxide were elevated on day 3 and day 7 by Isatis administration in the diabetic wound tissue. This data suggests that, for the first time, I. glauca subsp. sivasica extract may have the potential to promote the impaired wound healing in patients with diabetes.

Keywords:
Collagen; Diabetes mellitus; *Isatis glauca subsp. sivasica*; Nitric oxide; Wound healing.

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INTRODUCTION

Plants are traditionally used for medicinal purposes in the treatment of many diseases. *Isatis L.* genus (Brassicaceae), locally known as çivitotu in Turkey, has about 31 species and 15 subspecies in Turkey [1]. *Isatis L.* members are folkloric plants and are used both as an indigo plant and as a medicinal plant [2].

The members of this genus contain secondary metabolites like isatin, indicant, indirubin, tryptanthrin, p-coumaric acid, quercetin, chlorogenic acid, p-OH benzoic acid, caffeic acid [2,3] and have antioxidant, antimicrobial, anti-inflammatory and antinociceptive properties [3,4]. These plants are used for rheumatism, asthma, ulcer, constipation, fever, tumor, eczema, bite and hemorrhoid [5-7]. In addition, it has been reported that I. glauca subsp. glauca, I. glauca subsp. iconia and I. tinctoria are traditionally used for the wound healing in Turkey [8,9].

Wounding is one of the oldest and basic health problems in human history. Since the human body heals wounds by itself as an innate ability, wound healing may be overlooked. However, it is a highly complex and sensitive physiological process consisting of intertwined stages (inflammation, proliferation, remodeling) [10]. Many factors can disrupt this process and decrease the quality of life by delaying or preventing healing. Diabetes mellitus is one of these factors and is a systemic disease characterized by hyperglycemia [11]. In diabetic patients, almost all stages of the wound healing process are damaged. The diabetic wound healing process is disrupted due to the complications such as reduced collagen and growth factors production, infection, changing cellular activities, oxidative stress, vascular diseases and neuropathy [11,12].

The collagen is the dominant protein of the extracellular matrix (ECM) in the dermal layer of the skin, and is a vital element of the wound healing process [13]. Additionally, nitric oxide (NO) is a significant message molecule synthesized by various cell types in the skin during the healing process [14]. It has been reported that collagen and nitric oxide levels decrease in diabetic wounds [15,16].

Alternative treatment methods have been investigated in order to promote and accelerate diabetic wound healing process. This study was carried out to evaluate

the phenolic compounds of I. glauca subsp. sivasica (locally known as Sivas çiviti) extract and examine the effects of the extract on collagen and nitric oxide levels and wound closure rates throughout diabetic healing process on day 3 and 7.

MATERIAL AND METHODS

Plant Materials

I. glauca subsp. sivasica (Davis) Yıldırımlı samples were collected from Kemah/Erzincan province (between Kemah-Erzincan roadside, 1300-1400 m) in Turkey and were identified by Dr. Mustafa Karaköse. The voucher specimens have been deposited at the Espiye Vocational School Herbarium, Giresun University (Voucher number: ESPH 20)

Preparation of the *Isatis* Extract

The collected and identified plant samples (aerial parts) were dried under shade. Powdered samples (5 g) were extracted with methanol (100 mL) using Soxhlet apparatus. After the filtration with Whatman Millipore filter paper, methanol was evaporated by using a rotary evaporator (The yields of the extract: 15.69% w/w from dried starting material).

Reverse phase-high performance liquid chromatography method with diode array detector (RP-HPLC-DAD) analysis

The analysis was obtained according to a previously described method [17]. The gradient program was set by using the binary solvent system (A: distilled water with 2% acetic acid, B: distilled water with 70% acetonitrile, initial condition 5% B, final condition 60% B, run time 26 min). The column was operated at temperature of 30 °C. The flow of mobile phase and injection volume were adjusted as 1.2 mL/min and 10 µL, respectively. The eluted 10 phenolic acid standards and 2 flavonoids were analyzed at 280 nm by using a reversed phase column (Table 1). The validation parameters such as limits of detection (LOD) and limits of quantification (LOQ) were calculated to present much more reliably quantified results about analyses. Briefly, the phenolic compounds of the *Isatis* extract were evaluated by RP-HPLC-DAD using the calibration and validation values of the studied standard phenolic compounds (Table 1).

Induction of the Diabetes Model

This study was conducted with the approval from Gazi University Local Ethics Committee for Animal Experiments (G.Ü.ET-15.052). 24 Wistar rats were accommodated in standard cages with rat food and water (at room temperature, normal light-dark cycle). The diabetes model was induced in all rats via single dose intraperitoneal freshly prepared streptozotocin (STZ) (Sigma, USA) solution injection (60 mg/kg in sodium citrate buffer, pH 4.5). Three days after STZ injection, glucose levels were determined by a commercial glucometer, and those with above 250 mg/dL were considered as diabetic.

Induction of Wound Model

The animals were anesthetized with IM ketamine and xylazine. The dorsum of the rat was shaved and cleansed. Full thickness excisional skin wounds (six per animal) were created by using a biopsy punch (8-mm, Acu-Punch, Acuderm, USA) in all rats. The animals were randomly divided into four groups under two main groups (control and *Isatis*).

1. Control day 3: Wounded, no treatment was applied, sacrificed at post wounding day 3 (n=6)

2. Control day 7: Wounded, no treatment was applied, sacrificed at post wounding day 7 (n=6)

3. *Isatis* day 3: Wounded, the *Isatis* extract was applied, sacrificed at post wounding day 3 (n=6)

4. *Isatis* day 7: Wounded, the *Isatis* extract was applied, sacrificed at post wounding day 7 (n=6)

The *Isatis* extract was dissolved in 500 mL of physiological saline and the dose was adjusted such that 50 mg/kg of plant extract was applied to each wound (approx. 1 mL solution) based on the weight of the rats. In the treatment groups, the *Isatis* extract was applied topically to wounds via a sterile pipette as a single daily dose until sacrifice. The animals were sacrificed under anesthesia at post wounding day 3 or day 7, and wound tissue samples were collected. The collected samples were stored at – 80 °C. Simultaneously, the punch samples representing day 0 were also collected from the animals of the same groups.

Measurement of Collagen Levels

The collagen assay kit (Biocolor, UK) was used to measurement of the total collagen amount (type I-V) in the wound tissues. Collagen levels were determined according to the manufacturer's protocol and Tsuda et al. [18]. Briefly, the collected samples were homogenized in pepsin-acetic acid solution overnight, and mixed with Sircol dye reagent. Finally, the absorbances were recorded spectrophotometrically at 540 nm.

Table 1. The calibration and validation values of the studied standard phenolic compounds

No	RT	Standarts	R^2	RSD%(RT)	RSD%(Area)	LOD (mgL^{-1})	LOQ(mgL^{-1})
1	3.72±0.006	Gallic acid	0.999	0.168	4.315	0.070	0.213
2	6.74±0.019	Protocatechuic acid	0.998	0.291	5.973	0.495	1.499
3	10.13±0.029	p-OH Benzoic acid	0.999	0.290	4.817	0.224	0.680
4	11.46±0.027	Chlorogenic acid	0.997	0.239	6.177	0.512	1.550
5	13.49±0.023	Vanillic acid	0.994	0.168	6.794	0.171	0.518
6	13.84±0.032	Caffeic acid	0.999	0.235	6.861	0.058	0.175
7	14.79±0.012	Syringic acid	0.999	0.082	5.116	0.096	0.290
8	16.41±0.010	p-Coumaric acid	0.999	0.061	2.935	0.005	0.014
9	16.63±0.012	Rutin	0.999	0.075	2.855	0.311	0.942
10	18.41±0.013	Rosmarinic acid	0.999	0.069	3.388	0.162	0.492
11	18.84±0.014	Benzoic acid	0.999	0.076	2.721	0.550	1.665
12	21.71±0.019	Quercetin	0.999	0.087	2.268	0.335	1.014

Determination of NO_x Levels

The levels of NO_x in the wound tissue were evaluated according to a previously described method [19]. NO_x levels were calculated by Griess reagent assay. Briefly, the collected samples were homogenized in phosphate buffer (pH 7), and mixed with Griess reagents (N-1-naphthylethylenediamine dihydrochloride and sulphanilamide). After, the absorbances were recorded spectrophotometrically at 540 nm. Sodium nitrite and sodium nitrate solutions were used as standards.

Determination of Wound Size and Wound Closure Rate (WCR)

The wounds were photographed during the healing process. The wound sizes and the wound closure rates are assessed and measured using the ImageJ software (NIH, USA). The WCR was calculated as follows:

WCR (%) = [(wound size day 0–wound size on day 3 or 7)/wound size day 0] × 100.

Statistical Analysis

The obtained results were presented as the mean ± standard deviation (SD), and were compared using one-way ANOVA post-hoc Tukey test. Results with $P<0.001$ were interpreted as statistically significant (SPSS version 16, IBM, USA).

RESULTS

Phenolic Compounds

The RP-HPLC-DAD analysis revealed that *Isatis* extract contained protocatechuic acid, rutin, vanillic acid, p-hydroxybenzoic acid, rosmarinic acid, p-coumaric acid, caffeic acid, and benzoic acid (Fig. 1). Benzoic acid (6.95 mg phenolic/g extract) and vanillic acid (2.88 mg phenolic/g extract) were major phenolics in *Isatis* extract (Table 2). Gallic acid, chlorogenic acid, syringic acid and quercetin were not detected (Table 2).

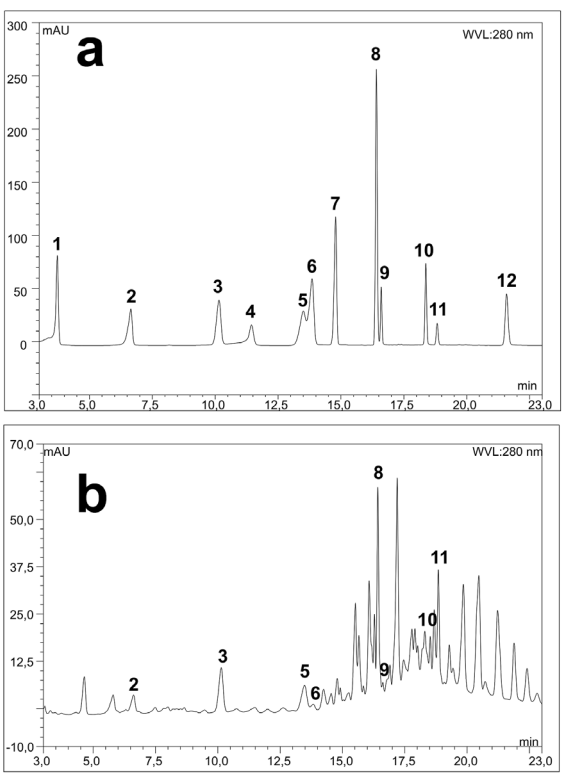

Figure 1. Chromatogram of I. glauca subsp. sivasica extract (a) standard phenolic compounds (b) Isatis (1) gallic acid (2) protocatechuic acid (3) p-OH-benzoic acid (4) chlorogenic acid (5) vanillic acid (6) caffeic acid (7) syringic acid (8) p-coumaric acid (9) rutin (10) rosmarinic acid (11) benzoic acid (12) quercetin

Table 2. Phenolic compounds of I. glauca subsp. sivasica extract

	Phenolic Compounds	*Isatis glauca subsp. sivasica*
		mg phenolic/ g extract
1	Gallic acid	n.d.
2	Protocatechuic acid	0.705
3	p-OH Benzoic acid	1.783
4	Chlorogenic acid	n.d.
5	Vanillic acid	2.879
6	Caffeic acid	0.103
7	Syringic acid	n.d.
8	p-Coumaric acid	1.158
9	Rutin	0.194
10	Rosmarinic acid	0.652
11	Benzoic acid	6.954
12	Quercetin	n.d.
	Total	14.432

n.d. = not dedected

3.2. Collagen and NO_x Levels

Topically administrated *Isatis* extract altered collagen and NO_x levels in diabetic wound tissue on both day 3 and day 7 compared to the control group as shown in Table 3. On day 3 and day 7, statistically increased collagen amount was determined in the *Isatis* group compared to the control group (P< 0.001) (Fig. 2). Similarly, NO_x levels in the *Isatis* group were also elevated significantly on day 3 and day 7 compared to the control group (P< 0.001) (Fig. 3).

Wound Sizes and WCRs

Fig. 4 shows the statistically significant decrease of wound size after *Isatis* extract administration on day 3 and day 7 compared to control group (P< 0.001). *Isatis* group

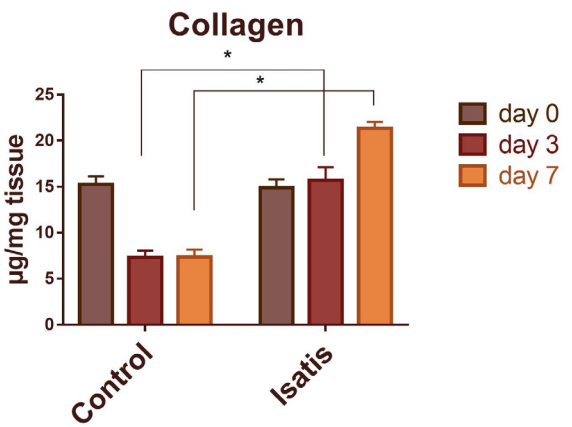

Figure 2. Comparison of *Isatis* and control groups in terms of collagen

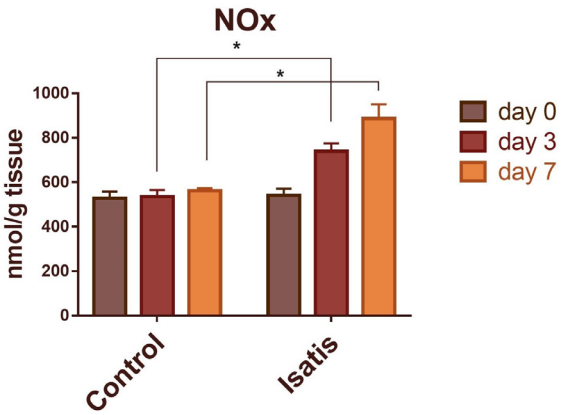

Figure 3. Comparison of *Isatis* and control groups in terms of NOx levels

displayed a higher WCR on day 3 and day 7 compared to control group (Table 4).

DISCUSSION

Since the wound healing is impaired in patients with diabetes, it is very important in terms of the life quality. New healing methods are increasingly gaining attention and are being researched in order to promote the diabetic wound healing process. Plant extracts have been used for this purpose since ancient times. This work aimed to investigate the effect of I. glauca subsp. sivasica extract on wound healing process of the STZ-induced diabetic Wistar rats. The I. glauca subsp. sivasica was chosen for this work because I. glauca and its various subspecies are traditionally used in the treatment of wounds as reported in ethnobotanical works [8,9].

The family of phenolic compounds are generally categorized into two class termed as phenolic acids and flavonoids. Besides ten phenolic acids, two flavonoids were analyzed for calibration and identification in this work. Our results showed that different ranges of concentration of pro-

Table 3. The collagen and NOx levels in diabetic wound tissue

	Collagen (µg/mg tissue)	*NOx (nmol/g tissue)*
Control group		
day 0	*15,24±0,87*	*528,26±30,41*
day 3	*7,32±0,73*	*535,43±30,38*
day 7	*7,36±0,81*	*562,10±11,27*
Isatis group		
day 0	*14,88±0,93*	*541,26±30,22*
day 3	*15,72±1,40**	*739,20±36,00**
day 7	*21,32±0,74**	*887,04±64,04**

*P<0.001 compared to control group on same day

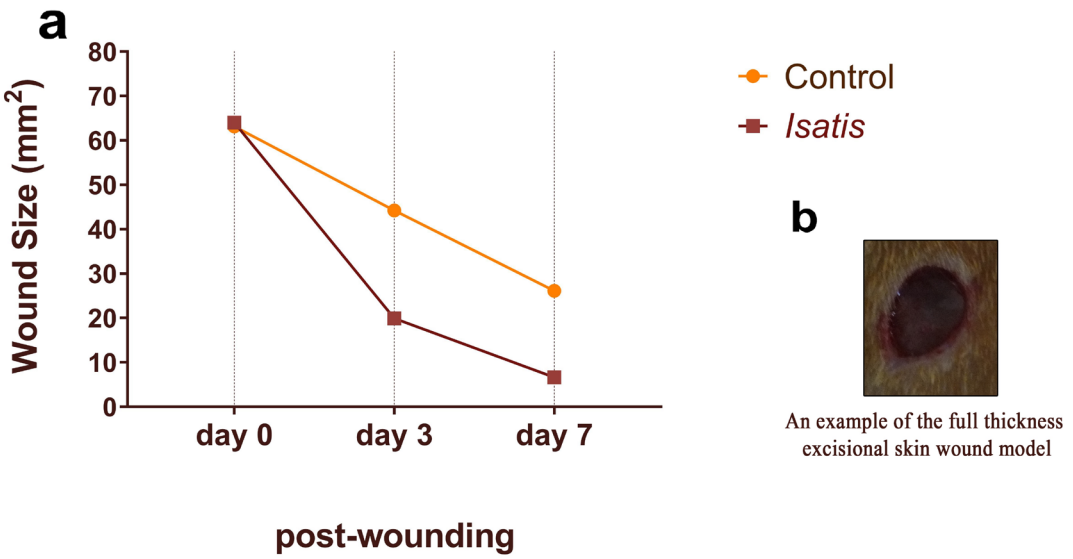

Figure 4. (a) Comparison of *Isatis* and control groups in terms of wound sizes depending on time (b) an example of the full thickness excisional skin wound model

	Wound size (mm²) and WCR (%)
Control group	
day 0	*63,15±3,48*
day 3	*44,22±2,41 (29,97 %)*
day 7	*26,11±1,72 (58,65 %)*
Isatis group	
day 0	*64,03±3,17*
day 3	*19,94±2,56* (68,86 %)*
day 7	*6,64±0,98* (89,63 %)*

*P<0.001 compared to control group on same day

Table 4. Wound sizes and WCRs during diabetic wound healing process

tocatechuic acid, rutin, vanillic acid, p-hydroxybenzoic acid, rosmarinic acid, p-coumaric acid, caffeic acid, and benzoic acid were contained in the *Isatis* extract. Phenolic compounds are commonly used in the treatment of various disorders thanks to its antioxidant and antimicrobial activity [17]. The *Isatis* extract can be considered as a source of bioactive compounds. The evaluated outputs are nearly similar to the phenolic compounds of the other members of the *Isatis* genus. Karakoca et al. [3] reported that I. floribunda contains phenolic components such as p-hydroxybenzoic acid, catechin, chlorogenic acid, caffeic acid, ferulic acid, quercetin, cinnamic acid and p-coumaric acid. In another study, Miceli et al. [20] showed that chlorogenic acid, sinapic acid, caffeic acid, ferulic acid, rutin, p-coumaric acid, apigenin-glucoside, isovitexin and vicenin-2 detected in I. tinctoria.

Collagen and NO are two important molecules that play a role in the complex wound healing process. Collagen is a major ECM component made up of amino acids (3 chains in triple helix form), and it accounts for about 75% of dry weight of the human skin. The synthesis, accumulation, deposition and degradation of collagen are important for proper wound healing process [21]. An important task of collagen is to increase the wound tensile strength by providing a structural support [13]. NO, an endogenous gasotransmitter, is synthesized from L-arginine by nitric oxide synthases (NOS), and has roles in the regulation of antimicrobial action, vascular homeostasis and inflammation during the wound healing process [16]. It has been demonstrated that both collagen and NO content decreased in the wounds of diabetes-induced animals and chronic wounds of diabetic patients [15,16]. These decreases may be one of the underlying causes of the damaged wound healing in diabetic patients. In our study, a statistically significant elevation in collagen and NO_x levels on days 3 and 7 were founded in the *Isatis* group compared with the control group.

However, there is no study showing the effects of I. glauca subsp. sivasica or another *Isatis* species on collagen and NO contents in the literature. These effects of *Isatis* may be due to its chemical composition. Kumar et al. [22] showed that the administration of vanillic acid increases NO levels in hypertensive rats. Additionally, it has reported that rutin enhances NO and collagen production in endothelial and fibroblast cells, respectively [23,24]. *Isatis* extract may also have increased the collagen and NO level due to the vanillic acid and rutin content (Table 2).

According to Kızıl et al. [25], palmitic, oleic, erucic, stearic, linoleic and linolenic acids were detected in the fatty acid composition of I. glauca. Lambertucci et al. [26] reported that palmitic acid induces NO production and of inducible nitric oxide synthases (iNOS) protein content in

muscle cells. Additionally, de Lima et al. [27] demonstrated that fatty acids (palmitic, stearic, oleic, linoleic acids) increase NO production in macrophages at low concentrations. Taken together, these results suggest that I. glauca subsp. sivasica extract can increase NO content in diabetic wound tissue through the composition of fatty acids and phenolic compounds. Furthermore, NO enhances the production and deposition of collagen during wound healing in non-diabetic or diabetic experimental models [28-31]. Hence, it can be stated that *Isatis glauca subsp. sivasica* extract contributes to diabetic healing process via increased collagen and nitric oxide levels.

Wound closure occurs due to the movement and proliferation of epithelial cells at the edge of the wound area (called as epithelialization). Diabetes mellitus causes non-closing (non-healing) wounds or impaired wound healing [11]. In our study, a statistically significant decrease in the wound sizes were measured in the *Isatis* group compared to the control group on day 3 and day 7 ($p<0,001$). In addition, WCRs detected in the *Isatis* group reached about 69% and 90%, in contrast to only 30% and 59% in the control group on day 3 and 7, respectively. The presented results suggested that this *Isatis* species accelerates the wound healing process supporting its traditional use.

CONCLUSION

This work shows that I. glauca subsp. sivasica extract can contribute to promote diabetic wound healing process by increasing collagen and NO levels and WCR on days 3 and 7. In this context, the I. glauca subsp. sivasica extract can be a potential therapeutic agent for the management of diabetic wounds.

CONFLICT OF INTEREST

Authors approve that to the best of their knowledge, there is not any conflict of interest or common interest with an institution/organization or a person that may affect the review process of the paper.

AUTHOR CONTRIBUTION

Kaan Kaltalioglu and Sule Coskun Cevher designed the study. KK performed all experiments. KK and SCC analysed the data. KK and SCC wrote the paper.

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