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Identification of major phenolic compounds of *Aloe vera* and its protective effect on ovaries under oxidative stress in diabetic rats

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ABSTRACT: The study investigated the major phenolic compounds of *Aloe vera* leaf (AVL) by the high-performance liquid chromatography (HPLC) method, and its protective effect on ovaries under oxidative stress of streptozotocin-induced diabetic rats. The study was conducted on control (untreated healthy rats; C), diabetes (untreated diabetic rats; D) and diabetes+*A. vera* treatment (diabetic rats treated with *A. vera*; D+A) groups. D+A group was given an ethanolic extract of *A. vera* (300 mg/kg) for 14 days. Major phenolic compounds of AVL were chlorogenic acid and rutin. alanine transaminase (ALT) and aspartate aminotransferase (AST) levels increased in D group compared to others groups ($P<0.01$). Alkaline phosphatase (ALP) and albumin levels were lower and higher in the C group than in other groups, respectively ($P<0.01$). In the D group compared to other groups, oxidative stress index (OSI) and total oxidant status levels increased, while total antioxidant status level decreased in both ovarian tissues and blood ($P<0.01$). Preantral and antral follicle counts decreased in the D group according to other groups ($P<0.01$) and atretic follicle count increased ($P<0.05$). Proliferating cell nuclear antigen (PCNA) expression level ($P<0.01$), and B-cell lymphoma-2-associated-X-protein (Bax; $P<0.01$) and tumor necrosis factor-alpha (TNF-α) expression levels ($P<0.05$) decreased and increased in group D according to other groups, respectively. In conclusion, treatment with AVL decreased OSI, improved follicular dynamics, and restored Bax, TNF-α and PCNA expression in diabetic rats.

KEYWORDS: *Aloe vera*; antioxidant; diabetes; HPLC; ovarian; rat.

1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia [1,2] and is associated with dysfunction, damage and failure in various organs [1]. Diabetes mellitus can cause dysfunction in the reproductive organs, including the ovaries [2]. The negative effect of diabetes mellitus on the ovaries occurs in different ways. One of them occurs as a result of the damage caused by diabetes in the insulin mechanism [3]. Another negative effect on the ovaries develops as a result of its causing oxidative stress [4]. Oxidative stress occurs when the level of oxidant substances exceeds the defense capacity of antioxidant substances. It damages cell components such as DNA, protein and lipid. Thus, it can lead to fertility problems or infertility [5]. The pathological effects of oxidative stress on the ovaries are caused by the destruction of granulosa cells, irregularities in oocyte development, disorders in follicular dynamics [6], increased risk of polycystic ovarian syndrome [3], disruption of the mechanism of steroidogenesis and cell apoptosis [5]. For these reasons, antioxidant substances have indispensable importance in the protection of ovarian and reproductive health. Antioxidants are obtained endogenously and exogenously. Exogenous antioxidant substances are very important because they are essential for the production of endogenous antioxidants. In addition, there are synthetic and natural types of exogenous antioxidant substances [7]. However, natural antioxidants are better [7,8] because many synthetic antioxidants can have toxic or

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mutagenic effects [7]. Plants are among the most important natural sources of exogenous antioxidants [9, 10]. Aloe vera (L.) Burm.f. is one of theplants with antioxidant content [11, 12] and is a perennial plant with succulent leaves belonging to the Asphodelaceae family [13]. The plant is well known for its use in food, food supplements, herbal medicines and cosmetics [14], and is among the few medicinal plants that have been on the agenda for a long time [11]. It is native to tropical and sub-tropicalAfrica regions. However, it is now cultivated worldwide, including in the mediterranean region, and is also one of the most economically important medicinal plants [15]. A. vera is described as an acaulescent herb, rarely with a stem thick and yellow when dry; leaves sessile, erect-spreading, lanceolate, often reddish at margins. The habitat of the plant is rocky slopes of old ruins [16]. The plant generally contains polysaccharides, lipids, sugars, proteins, minerals and phenolic compounds [17, 18], and its major phytoconstituents are anthraquinones, naphthalenones, proteins, polysaccharides, enzymes and organic acids. In addition, anthraquinones are the main bioactive compounds in the A. vera leaf [18]. It has been reported that this plant has wound healing, anti-ulcer, anticancer, anti-diabetic, antioxidant and anti-inflammatory pharmacological effects [17]. Moreover, A. vera is traditionally used for mild fever, wounds and burns, gastrointestinal disorders, diabetes, sexual vitality and fertility, cancer, immunemodulation, AIDS, and various skin diseases [19]. Furthermore, it is known for anti-diabetic effects [1,20] and its antioxidant effect [11,12].

The present study hypothesized that *A. vera* could alleviate the devastating effects of diabetes on the ovaries in rats. As a current approach, we aimed to determine the primary substances of phenolic compounds in ethanolic extract of *Aloe vera* leaf (AVL) by the high-performance liquid chromatography (HPLC) process, and to investigate its protective effect on the ovaries by biochemical, histopathological and immunohistochemical methods in experimental diabetic rats.

2. RESULTS

2.1. Plant materials

2.1.1. Analytical performance of the developed method

Chlorogenic acid (CA) and rutin (RU) as the major phenolic compounds were identified and quantified in *Aloe vera* leaf (AVL) under optimized chromatographic conditions (Figure 1). All results of the validation studies obtained for CA and RU are summarized in Table 1. The linear working range for each target compounds was performed by plotting the peak areas *vs.* different phenolic compound concentrations with three replicate injections. The linearities for CA and RU were determined in the concentration range of 10 – 200 µg mL^{-1} and 250 – 2000 µg mL^{-1}, and the corresponding LOD and LOQ values were calculated as 1.08 and 3.27 µg mL^{-1} for CA, 25.8 and 78.2 µg mL^{-1} for RU, respectively.

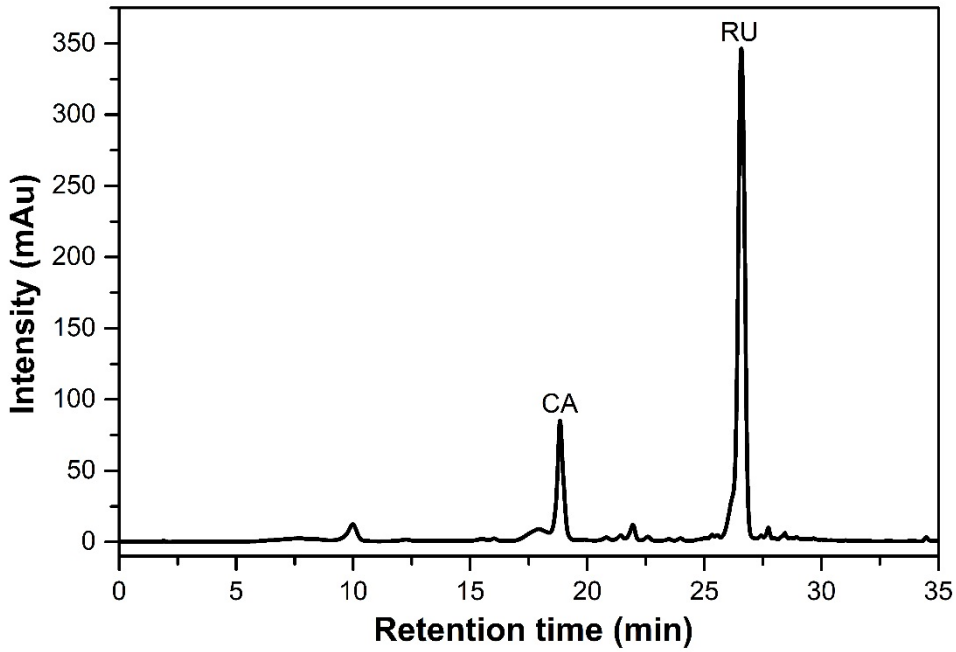

Figure 1. HPLC chromatogram of ethanolic extract of *Aloe vera* leaf (AVL). CA:Chlorogenic acid, RU: Rutin

Table 1. Validation parameters obtained at developed HPLC method for CA and RU (n=3).

Parameters	CA	RU
Linearity ranges (µg mL^{-1})	10 -200	250- 2000
Slope ± SD[a]	11.388 ± 0.046	3.2929 ± 0.011
Intercept ± SD[a]	12.378 ± 3.72	265.26 ± 25.75
Determinationcoefficient (R^2)	0.9997	0.9922
LOD (µg mL^{-1})	1.08	25.8
LOQ (µg mL^{-1})	3.27	78.2
Intra-day precision (RSD,%)[b]	1.02	1.97
Inter-day precision (RSD,%)[b]	1.08	0.41

[a]SD, standard deviation; [b]RSD, relative standard deviation

The precision of the proposed high-performance liquid chromatography (HPLC) method was determined by analyzing the intra-day and inter-day repeatability for CA and RU on same day and three consecutive days. The corresponding RSD% values for intra-day and inter-day were less than 2%, confirming that the developed HPLC method is a sufficiently precise for analyzing the CA and RU. On the other hand, the accuracy of the HPLC method was evaluated by calculating the average recoveries of CA and RU in AVL. For this purpose, AVL was spiked with a known concentration of CA and RU standard solutions and then analyzed by developed HPLC method under optimized experimental conditions. The average recoveries for CA and RU were calculated in the range of 98.5-103.9% with the low RSD% values below 3% (Table 2).

Table 2. Recovery analysis results of the proposed HPLC method for CA and RU.

	CA	RU
Added (µg mL^{-1})	75	1500
Found (µg mL^{-1})	77.9	1476.9
Recovery (%)[a]	103.9	98.5
RSD (%)	2.76	2.78

[a]Average of three replicate measurements

2.1.2. Determination of CA and RU contents in AVL

The developed HPLC method was used to determine the amounts of CA and RU in AVL. The sample extracts were diluted to be in the linear working ranges for CA and RU, and then analyzed by HPLC with five replicate measurements. Fig. 1 demonstrates the representative chromatogram of AVL, in which the peaks associated with phenolic compounds analyzed are labeled. As seen in Fig. 1, AVL contains two well-separated major phenolic compounds (CA and RU) at of 18.6 and 26.8 min, respectively. The contents of CA and RU in AVL were calculated as 8 and 150 µg per mg dry extract, respectively.

2.2. Animals

2.2. 1. Biochemical parameters

There was no significant difference in creatine (CRE) level between the groups ($p>0.05$). alanine transaminase (ALT) and aspartate aminotransferase (AST) levels increased significantly in the D group compared to C and D+A groups ($P<0.01$), but there was nosignificant difference between C and D+A groups in terms of ALT and AST levels ($p>0.05$). Alkaline phosphatase (ALP) and albumin levels were lower and higher in group C compared to other groups ($P<0.01$), respectively, but there was a similarity between the D and D+A groups in terms of these values ($p>0.05$). These results are detailed in table 3.

2.2.2. Oxidative stress in blood

Total antioxidantstatus (TAS) level decreased significantlyin the D group compared to C ($P<0.05$) and D+A ($P<0.01$) groups, and it increased in the D+A group compared to other groups ($P<0.01$). It was found that total oxidantstatus (TOS) and oxidative stress index (OSI) levels were higher in D groups than in C ($P<0.01$) and D+A ($P<0.05$; $P<0.01$) groups. On the other hand, a decrease in TOS and OSI levels were observed in D+A group compared to D group ($P<0.05$; $P<0.01$).

Table 3. Concentrations of some biochemical parameters in blood serum of all groups.

Parameters	Groups			
	C (Mean ± SD)	D (Mean ± SD)	D+A (Mean ± SD)	P
CRE (mg/dL)	0.35 ± 0.07[a]	0.32 ± 0.08[a]	0.37 ± 0.06[a]	p>0.05
ALT (IU/L)	78.14 ± 9.49[a]	223.71 ± 62.17[b]	125.14 ± 43.33[a]	p<0.05
AST (IU/L)	260.29 ± 37.37[a]	454.43 ± 97.26[b]	213.00 ± 32.45[a]	p<0.01
ALP (IU/L)	129.86 ± 10.09[a]	779.14 ± 170.76[b]	538.43 ± 212.89[b]	p<0.01
Albumin (g/dL)	6.71 ± 0.53[a]	5.38 ± 0.17[b]	5.44 ± 0.41[b]	p<0.01

a,b: Different letters at the same line indicate the statistical difference (P<0.05).

2.2.3. Oxidative stress in ovaries

TAS level was lower in the D group than in others (P<0.01), but it increased significantly in D+A group compared to D group (P<0.01). On the other hand, a similarity was observed between the C and D+A groups (P>0.05). TOS and OSI levels were significantly higher in D group compared to the other groups (P<0.01) and lower in the D+A group than in the D group (P<0.01). However, these parameters were similar between C and D+A groups (P>0.05). Oxidative stress parameters in ovaries and blood are given in detail in table 4.

Table 4. Comparison of the TAS, TOS and OSI levels in blood serum and ovarian tissues of C, D and D+A groups.

		Groups			
	Parameters	C (Mean ± SD)	D (Mean ± SD)	D+A (Mean ± SD)	P
Blood	TAS (mmol/L)	1.40 ± 0.07[a]	1.17 ± 0.07[b]	1.66 ± 0.09[c]*	p<0.05
	TOS (µmol/L)	5.18 ± 2.46 [a]*	16.62 ± 4.13[b]	10.51 ± 0.39[c]	p<0.05
	OSI	0.36 ± 0.16 [a]	1.44 ± 0.43[b]*	0.63 ± 0.04[c]	p<0.05
Ovarian	TAS (mmol/L)	0.58 ± 0.13 [a]	0.29 ± 0.09[b]	0.53 ± 0.03[c]	p<0.01
	TOS (µmol/L)	2.08 ± 0.04 [a]	4.31 ± 0.43[b]	2.14 ± 0.22[c]	p<0.01
	OSI	0.37 ± 0.07 [a]	1.62 ± 1.21[b]	0.47 ± 0.11[c]	p<0.05

TAS: Total antioxidant status, TOS: Total oxidant status, OSI: oxidative stress index. a,b,c: Different letters at the same line indicate the statistical difference (P<0.05). *P<0.01 C: control, D: Diabetes, D+A: diabetes + AVL treatment.

2.2.4. Ovarian weight

Ovarian weightwassignificantlylowerin the D group (0.03 ± 0.01 g) than in the D+A (0.05 ± 0.01 g) and C (0.06 ± 0.01 g) groups (P<0.01), and it increased in the D+A group compared to D group (P<0.01). However, there was a similarity between the C and D+A groups in terms of ovarian weight (p>0.05).

2.2.5. Ovarian histomorphology

In C group the ovarian follicles were observed as distributed randomly within the ovarian tissue and the morphology of the ovarian follicles were normal with regular oocyte and surrounding granulosa cells. In D group, we observed some pathological changes in ovarian tissues and follicles. Pycnotic granulosa cells in ovarian follicles of this group were more densely distributed and degenerated oocytes were more widespread. On the other hand, the atretic follicle findings of D+A group were more similar to the C group. Medullary region of the D group was more edematous and lymphocyte infiltration in this group was denser than the C and D+A groups. Germinal epithelium, stromal cells, luteal cells, vascular structures and hilum morphology in all of the 3 groups were observed as similar and normal. It was determined that the numbers of primordial and primary follicles were similar in all groups (P>0.05). The numbers of preantral and antral follicles decreased significantly in D and D+A groups compared C group (P<0.01). However, the numbers of these follicles increased significantly in D+A group compared to D group (P< 0.01). It was observed that number of atretic follicles was the lowest in C group compared to D (P<0.05) and D+A groups (P<0.01).

Besides, atretic follicles decreased significantly in D+A group compared to D group (P<0.01). The numbers of follicles follicle obtained from 3 groups are detailed in table 5, and representative micrographs of H&E stained samples are were shown in Figure 2.

Table 5. Comparison of the number of primordial, primary, preantral, antral and atretic follicles in C, D and D+A groups.

	Groups			
Follicles	C (Mean ± SD)	D (Mean ± SD)	D+A (Mean ± SD)	P
Primordial	18.71 ± 1.89[a]	16.00 ± 4.97[a]	17.14 ± 2.27[a]	>0.05
Primary	9.29 ± 0.95[a]	6.86 ± 2.41[a]	8.43 ± 0.98 [a]	>0.05
Preantral	8.14 ± 1.21[a]	3.57 ± 0.53[b]	5.86 ± 0.69[c]	<0.01
Antral	5.14 ± 0.38[a]	1.71 ± 0.49[b]	3.57 ± 0.53[c]	<0.01
Atretic	1.86 ± 0.38[a]	6.14 ± 1.07[b*]	3.14 ± 0.90[c]	<0.05

a,b,c: Different letters at the same line indicate the statistical difference (P<0.05). *P<0.01. C: control, D: Diabetes, D+A: diabetes + AVL treatment.

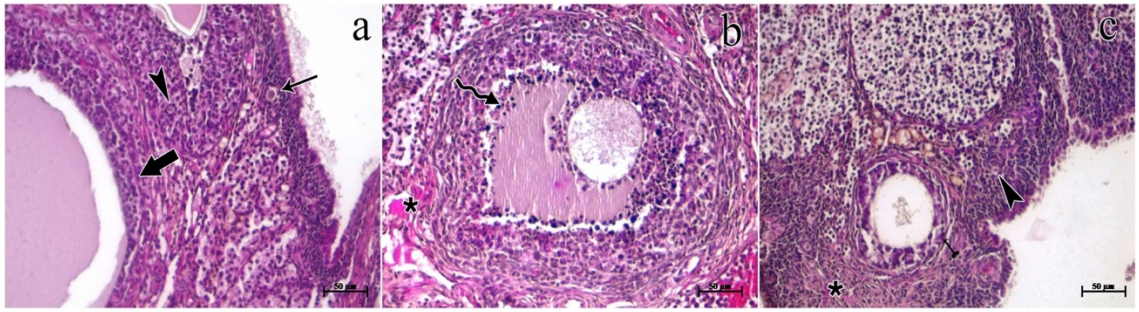

Figure 2. Representative H&E stained micrographs of control (**a**), diabetes (**b**) and diabetes + AVL (**c**) groups. **a;** Normal morphological features in primordial (arrow head), primary (arrow) and antral (thick arrow) follicles in control group. **b;** Increase in apoptotic bodies in ovarian follicles (curved arrow) and increased stromal edema (asterix) in diabetes group. **c;** Normal morphological structure in primordial (arrow head), and pre-antral (arrow with tail), decreased edema (asterix) in diabetes + AVL. **Staining:** Hematoxylin & eosin. **Bar:** 50 µm.

2.2.6. Immunohistochemistry

Immunohistochemistry sections were evaluated for immuno-expression of B-cell lymphoma-2-associated-X-protein (Bax), expression level, and tumor necrosis factor alpha (TNF-α) and proliferating cell nuclear antigen (PCNA). Varying immunopositivity of Bax was observed in granulosa cells, oocytes and stromal cells in C, D and D+A groups. Bax immunoexpression level was significantly higher in the D group compared to the C and D+A groups (P<0.01). However, it was similar between D+A and C groups (P>0.05). we observed that TNF-α was predominantly distributed in the ovarian stroma and hilum. Its immunopositivity was very low in ovarian follicles. TNF-α level was lower in the C group compared to D and D+A groups (P<0.01). On the other hand, it was found that TNF-α level decreased significantly in D+A group than in D group (P<0.05). The distribution of PCNA immunopositivity was observed nuclei of granulosa cells. In addition, stromal cells nuclei in ovarian medulla and hilum were predominantly negative for PCNA in all groups. PCNA expression positive granulosa cells index was significantly higher in C group compared to D and D+A groups (P<0.01). At the same time, it increased significantly in D+A group compared to D group (P<0.01). Detailed results of Bax, TNF-α and PCNA are given in table 6, and representative micrographs for immunohistochemistry samples are shown in Figure 3.

Table 6. Comparison of results of Bax, TNF-α and PCNA in C, D and D+A groups.

	Groups			
Paramaters	C (Mean ± SD)	D (Mean ± SD)	D+A (Mean ± SD)	P
Baxlevelscore	0.33±0.48[a]	1.57±0.68[b]	0.67±0.58[a]	<0.01
TNF-α levelscore	0.24±0.44[a]*	1.33±0.48[b]	0.95±0.50[c]	<0.05
PCNA (%)	69.57 ± 8.66[a]	30.57 ± 5.13[b]	45.43 ± 6.68[c]	<0.01

a,b,c: Different letters at the same line indicate the statistical difference (P<0.05). *P<0.01. C: control, D: Diabetes, D+A: diabetes + AVL treatment.

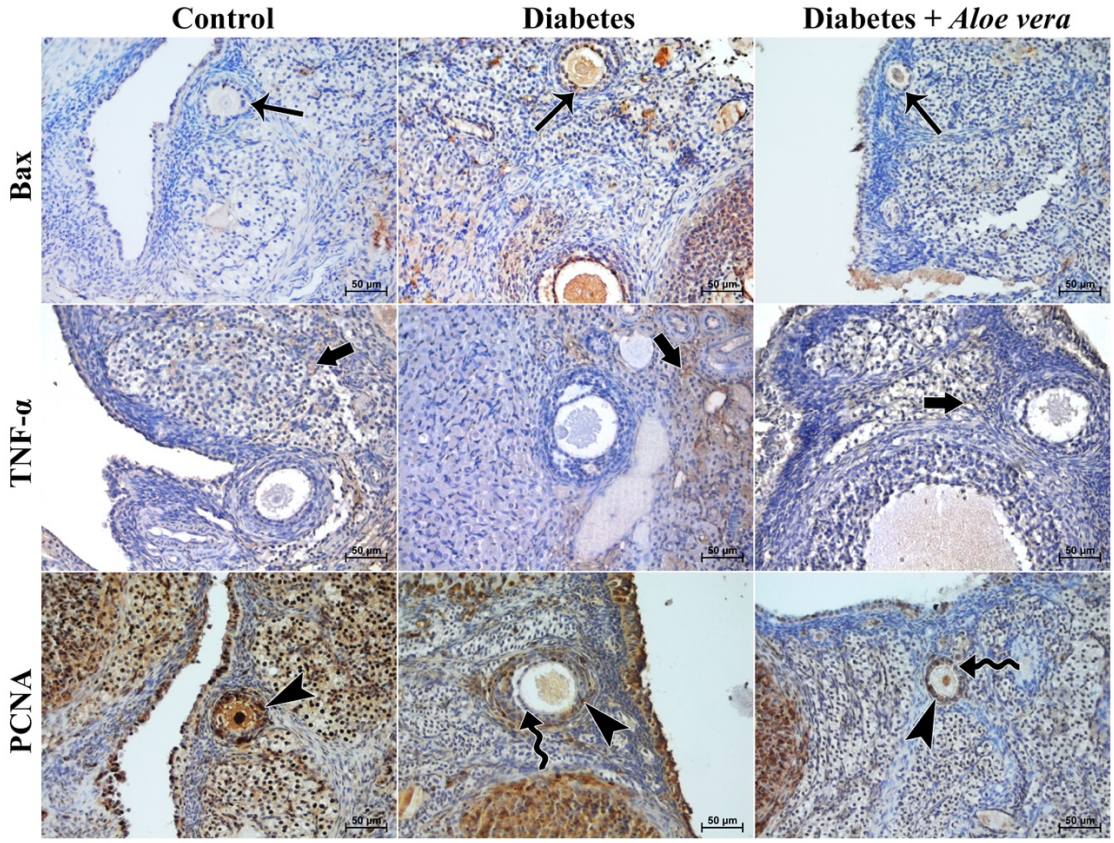

Figure 3. Representative micrographs of Bax, TNF-α, PCNA immunohistochemistry in control, diabetes and diabetes + AVL groups. Bax immunopositivity (arrow) in ovarian follicles, TNF-α immunopositivity observed predominantly in oviarian stroma (thick arrow) in a varying level, PCNA immunopositivity (arrow head) and negative reaction (curved arrow) in ovarian granulosa cells. **Staining:** Bax, TNF-α, and PCNA immunohistochemistry. **Bar:** 50 µm.

3. DISCUSSION

The present study investigated the protective effect of *A. vera* on the ovaries in streptozotocin (STZ)-induced diabetic rats. To reveal this comprehensively, major phenolic substances responsible for the antioxidant activity were determined by high-performance liquid chromatography (HPLC) method, and control (untreated healthy rats), diabetes (untreated diabetic rats) and diabetes+*A. vera* treatment (diabetic rats treated with *A. vera*) groups of female rats were formed. The D+A group was given an ethanolic extract of *A. vera* at dose of 300 mg/kg for 14 days. The reason why only one dose of the 300 mg treatment was used in the study was that previous studies had determined the antidiabetic and antioxidant effects of similar doses of aloe vera treatment in rats [2, 11, 21].

It is well known that *A. vera* samples contain various phenolic compounds, which are the characteristics for their identification [22]. In this context, chlorogenic acid (CA) and rutin (RU) were determined as the major phenolic compounds by HPLC in *Aloe vera* leaf (AVL). The developed HPLC method was widely validated by evaluating the various analytical parameters such as linearity, the limit of

detection (LOD), the limit of quantification (LOQ), accuracy, precision, etc. according to the International Council on Harmonization (ICH) guidelines [23]. All results demonstrated the sensitivity and reliability of the developed HPLC method for the determination of CA and RU in AVL. It has been reported that diabetes impairs the structure and function of the ovaries in rats [2]. Furthermore, it is associated with decreased ovarian reserve [4], impaired steroidogenesis and folliculogenesis [24]. Similarly, Shima et al. [2] reported that diabetes affects follicular dynamics, reduces the number of follicles and impairs follicle development. Moreover, it causes histopathological disorders and structural changes in the ovaries. These negative effects on the ovaries are accompanied by dysfunctions of various organs because diabetes affects health in many ways [1, 2, 24], and it is known that it damages liver functions and affects the level of many serum biochemical parameters [25]. Similarly, in this study, diabetes deteriorated in some biochemical parameters including alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and albumin. While other parameters increased, albumin level decreased, indicating that diabetes impairs glomerular filtration in the kidneys. Moreover, diabetes caused a decrease in the number of preantral and antral follicles and an increase in the number of atresia follicles by causing negativities in the structure of the ovaries in D group compared to C group. However, it did not cause any change in the number of primordial and primary follicles. Considering these results, it is concluded that diabetes does not have adverse effects on the emergence of new follicles from the follicular pool, but impairs follicular development. On the other hand, diabetes adversely affected the ovarian tissue mass in D group compared to C group. This confirms that it causes tissue destruction in the ovaries. When examined immunohistochemically, it was determined that diabetes caused serious disorders in terms of B-cell lymphoma-2-associated-X-protein (Bax), tumor necrosis factor alpha (TNF-α) and proliferating cell nuclear antigen (PCNA) expression in the ovaries. Bax is known as a proapoptotic protein, and Bax expression increases when apoptosis increases in cells. Therefore, in immunohistochemical studies, the Bax expression is a powerful predictive tool in determining the level of pathology in cells [26]. On the other hand, TNF-α is a proinflammatory cytokine and controls tissue homeostasis by coordinating the cell survival and death cycle [27]. It is secreted by defense cells, triggering the repair mechanism for tissue damage, and its expression increases with inflammation due to pathological disorders in cells. So, its level is closely related to the extent of cellular pathology [27]. PCNA expression level is associated with DNA replications in cells [28]. PCNA has the ability to regulate the cellular cycle and is involved in DNA repair mechanism [29]. It was reported that PCNA expression increases with proliferation of granulosa cells during follicular development. However, it has been reported that PCNA expression decreases [30], while apoptosis of granulosa cells increases during follicular atresia [31]. According to the results, the Bax, TNF-α and PCNA results are supported by the above information, because while follicular atresia increased in group D compared to C group, PCNA expression level, and Bax and TNF-α expression levels decreased and increased, respectively. It has been suggested that these destructive effects on the ovaries are due to the oxidative stress and hyperglycemia caused by diabetes [2]. Diabetes causes deterioration in energy metabolism and as a result of the cells not using glucose in the blood, lipolysis is triggered and used in energy production. During this process, excessive levels of reactive oxygen species are produced and oxidative stress occurs. Therefore, it is understood that oxidative stress is a condition that should not be ignored for combating against diabetes. Many researchers have already reported that oxidative stress destroys ovarian functions and disrupts follicular dynamics [8, 32]. In the present study, diabetes caused an increase in total oxidant status (TOS) level with a decrease in total antioxidant status (TAS) level in both ovarian tissue and blood. It also increased oxidative stress index (OSI) in both the ovary and blood in the D group. This clearly revealed that the devastating effects of diabetes on the ovaries were caused by oxidative stress and hyperglycemia. On the other hand, it is known that one of the most effective strategies to combat the negative effects of oxidative stress and diabetes is antioxidant supplementation. There are several exogenous sources of antioxidants. However, natural antioxidants are better because of their low toxicity [7, 8]. On the other hand, natural antioxidants are thought to have low side effects because they do not have additives and are natural ingredients. Also, Pokorný [33] as noted, natural antioxidants are considered safe due to traditional use, but if they are to be consumed in significantly higher amounts than conventional use, toxicity should be tested. In our study, we used *A. vera,* which is a natural antioxidant source, due to its strong antioxidant properties [12] as well as its anti-diabetic effects [20]. In this study, as a result of HPLC analysis of *A. vera*, it was determined that the main substances responsible for the activity were CA and RU, which have phenolic properties. It was reported that phenolic compounds have strong antioxidant properties [34]. Thus, the antioxidant properties of the *A. vera* used in our study were confirmed. Antioxidant substances have improving effects on folliculogenesis [35]. Similarly, Zhong&Zhou [8] stated that plant-derived antioxidants substances increased follicular activity and total follicle count in ovaries. Similarly, our study found a significant increase in the antral and preantral follicle counts in D+A Group compared to D Group. Also, the number of atretic follicles decreased in D+A Group compared to D Group.

However, the number of primordial and primary follicles was not affected in any group. Thus, it is concluded that neither diabetes nor *A. vera* affect the formation of new follicles from the ovarian follicular pool. One of the previously published studies indicated that streptozotocin induced diabetes increases Bax expression in ovaries of mice, and apoptotic upregulation in ovaries of hyperglycemia can be alleviated with the administration of plant ingredients which have antioxidant activity [36]. Bax is a cytosolic pro-apoptotic protein, and until today numerous studies reported the correlation between increased oxidative stress and pro-apoptotic Bax expression [37]. From this perspective, our results in D+A group is consistent with the literature that indicates downregulated oxidative stress goes with the decreased Bax immunoexpression along. On the other hand, previously published aricles reported increased TNF-α expression both in serum and tissues of experimental diabetic aimals [38, 39]. Results of our study indicated that *A. vera* administration downregulates tissue expression TNF-α in D+A group compared to D group. We believe this activity is regulated with anti inflammtory property of *A. vera* extract [40]. Level of PCNA expression is upregulated during granulosa cell proliferation, however, PCNA expression gradually decreases with increasing follicular atresia [30]. A previous study reported that antioxidant substances improve the functions of granulosa cells during folliculogenesis [29]. Similarly, this study revealed that *A. vera* with antioxidant properties improved PCNA expression in D+A group compared to D group.

4. CONCLUSION

The devastating effects of diabetes on the ovaries were caused by oxidative stress and hyperglycemia. The major phenolic compounds of *A. vera* were chlorogenic acid and rutin. Treatment with ethanolic extract of *A. vera* leaf had improving effects on oxidative stress, follicular dynamics and ovarian tissue in female diabetic rats. Moreover, it immunohistochemically recovered the expression of B-cell lymphoma-2-associated-X-protein (Bax), tumor necrosis factor alpha (TNF-α) and proliferating cell nuclear antigen (PCNA) in diabetic rats. Nevertheless, we think further studies on *A. vera* should be performed and its effect on the ovaries is needed to investigate more comprehensively.

5. MATERIAL AND METHODS

5.1. Ethical approval

This study was approved by the Animal Experiments Local Ethics Committee of Dicle University, Turkey (Approval number: 04/03/2021-36188). In this protocol, there is no in vitro or another alternative method that could prevent the use of experimental animals for the present study. The numbers, management and use of experimental animals in present study were managed following the European Community Guidelines on care and ethical use of lab animals (Directive 2010/63).

5.2. Plant materials and extraction procedure

The plant material was obtained from a local region in Turkey. Then the ethanolic extract was prepared by the maceration method according to standard procedures [41, 42]. Yield (%) was calculated as g extract from 100 g dry plant. A voucher specimen of *A. vera* was deposited in the Ankara University Faculty of Pharmacy Herbarium (AEF 30758) in Turkey.

5.3. Experimental section

5.3.1. HPLC-UV/DAD conditions

Reversed-phase high-performance liquid chromatography (HPLC) analysis of *Aloe vera* leaf (AVL) was performed by Agilent 1100 Series chromatography system equipped with a diode array detector. The HPLC method was developed by the authors for the assay of chlorogenic acid (CA) and rutin (RU) in aloe vera leaf extract. Chromatographic separation of phenolic compounds were carried out using a Supelco® Lichrospher RP-C18 column (150 mm x 4.6 mm x 5 µm) in a mobile phase consisting of acetonitrile (A), methanol (B) and 0.1% phosphoric acid (C) with the following gradient elution system: 5:95% (v/v, A: C) at 0-10 min, 10: 90% (v/v, A: C) at 10-15 min, 15:15:70% (v/v/v, A: B: C) at 15-25 min, 20:20:60% (v/v/v, A: B: C) at 25-30 min, 30: 30: 40% (v/v/v, A: B: C) at 30-35 min with a flow rate of 1 mL min^{-1}. The chromatograms were monitored at a wavelength of 330 nm. Column temperature was maintained at 25 °C, and the injection volume was applied as 10 µL.

5.4. Animal and management

In this study, 21 female Wistar Albino rats were used. The rats were obtained from the Dicle University Experimental Research Center, and they were housed at 12 hours light, 12 hours dark and 23°C with constant humidity. The rats were fed a standard pellet diet and had free access to water.

5.4.1. Groups and experimental design

The rats were randomly divided into three experimental groups; control (C; n = 7), diabetes (D; n = 7) and diabetes+ AVL treatment (DA; n = 7). Then, the rats were weighed and each group was placed in separate cages.

Each group underwent experimental treatment according to the following methods:

- In the C group: Rats received no treatment.
- In the D group: Diabetes induced by streptozotocin (STZ) as previously described [2, 43]. Three days after STZ administration, blood glucose levels were measured with a glucometer from the blood samples taken from the tail veins. Rats with blood glucose levels higher than 240 mg/dL were considered experimental diabetic. The rats in this group did not receive any other treatment.
- In the D+A group: Experimental diabetes was induced in the same way as D group. Then, each diabetic rat in D+A group were treated orally with AVL at dose of 300 mg/kg/day for 14 days. The indicated dose of ethanolic extract was administered to each rat by dissolving it in 1 ml of water.

5.4.2. Sample collection and tissue preparation protocol

At the end of the study, all rats in C, D and D+A groups were anaesthetized with intramuscular xylazine and ketamine administrations in same period. Blood samples were collected by intracardiac method into vacutainer tubes with clot activator (Hema&Tube®, Italy), centrifuged at 3000 rpm for 10 min, the serum samples were immediately harvested, and stored at -80 °C until analysis of total antioxidant status (TAS), total oxidant status (TOS), creatine (CRE) alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and albumin. After blood collection procedures, the animals were euthanized under general anesthesia. In all rat, right and left ovarian tissues were removed in its entirety by making a ventral midline incision. One ovarium of each rat was weighed and fixed in 10% neutral buffered formalin solution. Then, these ovaries were dehydrated through increasing alcohol series and clarified using xylene. Other ovaries were ultrasonically homogenized with the working solution using a mechanical homogenizer device (Fisherbrand™ Model 50 Sonic Dismembrator), centrifuged at 3000 rpm for 5 min, super natants were separated and preserved at -80°C until TAS and TOS analysis. The fixed ovaries were embedded into paraffin blocks. Subsequently, serial sections of 5 µm thickness were obtained using the rotary microtome, with a distance of 45 µm between the sections. Five slides of each ovary were stained with hematoxylin and eosin (H&E) by standard procedures [44], and used for follicle examinations [10]. Three slides of each ovary were immunohistochemically stained with proliferating cell nuclear antigen (PCNA) expression level, B-cell lymphoma-2-associated-X-protein (Bax) and tumor necrosis factor alpha (TNF-α) antibodies, used for immunohistochemical evaluations.

5.4.3. H&E staining and immunohistochemistry

Obtained sections were deparaffinized in two series of xylene, then rehydrated with decreasing alcohol series. The sections were brought to the distilled water and stained with H&E [44]. Ovarian tissues for immunohistochemistry staining were prepared as previously described [45]. For this purpose, sections were washed in two series of PBS after distilled water. Antigen retrieval was performed in citrate buffer (Ph: 6.0) on a hot plate until the sub-boiling temperature was reached. The samples were brought to the room temperature and incubated in 3% H_2O_2 dissolved in methanol for 15 minutes. Non-specific binding of antibodies was blocked with Ultra V Block (Thermo Scientific, MA, USA – cat. no: TA-125-UB). Then, the Bax (Santa cruz, TX, USA - cat. no: sc-7480), TNF-α (Santa cruz, TX, USA - cat. no: sc-52746) and PCNA (Santa cruz, TX, USA - cat. no: sc-25280) antibodies were prepared by diluting 1/300, 1/300, 1/250, and they were dropped on the sections. After these procedures, the sections were left overnight at +4 °C. Thus, the incubation period was completed and the sections were washed in two series of PBS. Further steps were performed with a ready to use of UltraVision Large Volume Detection System: anti-Polyvalent, HRP (Thermo Scientific, MA, USA – cat. no: TA-125-UDX). All steps were performed according to manufacturer's instructions. DAB chromogen (Thermo Scientific, MA, USA – cat. no: TA-125-HD) was used for signaling development and samples were counterstained with hematoxylin. Finally, all sections were mounted with entellan and examined under a camera attached light microscope system.

5.4.4. Quantification of immunohistochemistry analyses

Immunohistochemically stained sections were quantified to assess the immune expression level of Bax, TNF-α and PCNA. All procedures were carried out under a light microscope at 400x magnification (Zeiss axio, Germany). Firstly, the h-score system was developed to semi-quantitatively evaluate the immunoexpression levels of Bax and TNF-a. Immunopositivity of Bax and TNF-α was determined by a scoring grade of 0 to 3, and the grading system was performed according to the following methods:

- Grade 0: Tissue section was totally negative.
- Grade 1: Tissue section was slightly immunopositive for interest protein.
- Grade 2: Tissue section was moderately immunopositive.
- Grade 3: Tissue section was densely immunopositive.

Three randomly selected areas from tissue section were used for scoring analyses. On the other hand, PCNA immunohistochemistry was analyzed quantitatively depending on whether DAB was positive or negative in ovarian granulosa cells. Thus, immunopositivity rate was obtained from total granulosa cells in each ovarian follicle. All immunohistochemistry data were analyzed statistically, and representative micrographs were captured with a camera attached light microscope (Zeissaxio, Germany).

5.4.5. Determination of TAS and TOS levels in blood serum and ovaries

TAS and TOS levels in supernatants of ovarian tissues and in blood serum were determined by colorimetric method using commercial kits (Rel Assay Diagnostics®, Gaziantep, Turkey).

5.4.6. Calculation of oxidative stress index (OSI)

OSI value was computed as the percentage ratio of TOS to TAS. Firstly, unit of TAS (mmol Trolox eq/L) was converted to µmol Trolox eq/L. Thus, the TAS and TOS units were made identical (µmol Trolox eq/L) and the OSI value was calculated with the following formula: TOS / TAS x 100 [46].

5.4.7. Follicle counting

H&E-stained sections were examined by a blind observer for follicle counting under a light microscope (Nikon H550L, Japan) at 20x objective. To avoid counting a follicle more than once, the follicles were counted if the intact nuclei of the oocytes were clearly observed [32]. Follicles were characterized as primordial, primer, preantral, antral and atretic according to the following definitions. Primordial follicle consisted of follicles containing an oocyte surrounded by a single layer of flattened granulosa cells [47]. The follicle containing a central oocyte surrounded by a single layer of cuboidal granulosa cells was termed the primary follicle [32]. Follicles with at least two or more layers of cuboidal granulosa cells and no antral cavity were defined as preantral follicles [32]. Follicle was classified as antral follicle if it had an antral cavity and numerous layers of cuboidal granulosa cells [32, 48]. On the other hand, when vacuolization in the follicles, pyknotic nuclei in the granulosa cells and shrinkage in the oocyte were observed, it was classified as atretic follicle [10, 32].

5.5. Statistical analysis

In this study, the size of each group was determined as seven according to the power analysis result using 80% power and 5% margin of error. All statistical calculations were performed using SPSS software (version 24.0; IBM, USA). Before the statistical analyses, the normality test was performed, therefore it was used Non-parametric Kruskal Wallis test. Multiple comparisons were performed with Tamhane's T 2 tests and $P<0.05$ was considered statistically significant. The results were shown as mean ± deviation error (SD).

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