

ORIGINAL RESEARCH

Resin extract obtained from Cilician fir (*Abies Cilicica*) inhibits glucose dependent inflammation in vitro

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Received: July 13, 2018. Revision: August 28, 2018. Accepted: September 06, 2018.

The potential anti-inflammatory efficacy of resin extract of *Abies cilicica* in glucose dependent inflammation and tumor necrosis factor alpha (TNF- α) induced inflammation models was investigated. Its effects on monocyte adhesion, gene expression levels of P-selectin, ICAM-1, VCAM1 and transendothelial migration for the two in vitro models were measured. Also, total flavonoid and total phenolic contents of the extract were determined.

Monocyte adhesion tests showed that the extract increased 100% inflammatory effect of TNF- α induced inflammation. On the other hand, it did not change number of adherent monocytes in glucose dependent inflammation model. Although the extract has trigger effect on monocyte adhesion, it did not change migration of leukocytes across ECV304 cells after administration of TNF α on ECV304 cells. The number of migrated monocytes was similar with only TNF α incubation experiment results. However, it significantly decreased monocyte migration in glucose dependent inflammation model. In our both experimental inflammation model, ICAM-1 expression significantly decreased. Although it is known that triggering effect of TNF- α on ICAM-1 expression, the content of of resin extract of *A. cilicica* prevented this effect. Phenolic antioxidant capacity of the extract are higher than its flavonoid contents. This study provides the first evidence that the extract inhibits glucose dependent inflammation. It may serve as an anti-inflammatory agent in the treatment of chronic inflammation caused by diabetes.

Keywords: *Abies cilicica*, Mezda gum, diabetes, inflammation, TNF- α , ICAM-1

INTRODUCTION

Inflammation is an immunological defense reaction against various foreign pathogens and systemic injuries. Not only activation of leukocytes, also a lot of inflammatory mediators secreted by various activated cells play a role this process. During inflammation, leucocyte rolling is mediated by members of the selectin family, and the expression of cell adhesion molecules (CAMs) such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) play a role monocyte/macrophage adherence and leucocyte transendothelial migration through vascular endothelium (1,2). VCAM-1 can also mediate rolling in early stage of inflammation (3).

On the other hand, chronic inflammation can lead to severe tissue damage and associated with many diseases such as atherosclerosis, arthritis, asthma, neurological disorders, diabetes, and cancer. Therefore, finding of new agents targeting to suppress of chronic inflammatory reactions is very important for the diseases (4).

One of the diseases is type 2 diabetes mellitus (T2D) and induces diabetic micro and macrovascular complications and atherosclerosis. (2). These vascular complications results in cardiovascular disease, chronic renal

failure, retinal damage, neuropathy and poor wound healing. In addition, it was shown that several vascular adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin increased during the early stage of T2D in many studies. However, molecular mechanisms of these vascular abnormalities in T2D are not clear (2,5).

During early stage of inflammation, adhesion molecules in endothelial cells are induced by pro-inflammatory factors such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-1 β (IL-1 β), and monocyte chemoattractant protein-1 (1, 6-11). TNF α also overexpressed in many neuroinflammatory conditions including multiple sclerosis, encephalitis, stroke, and HIV encephalitis (12).

Various anti-inflammatory drugs such as Pentoxifylline, metformin, aspirin and low dose statin drugs are used in the treatment of many chronic inflammatory diseases (13-15). These drugs repair the damaged tissue by restricting the vascular and cellular responses resulting from the inflammation, and inhibit proinflammatory cytokine production and leukocyte activation (16-18). Moreover, natural extracts of several medical plants including *Streptocaulon juvenas* root, *Taraxacum officinale*, *Thevetia peruviana* were examined for anti-inflammatory effects (19-21).

Abies cilicica extracts have been shown to be anti-tumoral, anti-microbial, anti-hypertension, cough suppressant, and in various studies. Mezda gum, the natural product of *Abies cilicica* contains essential oils having antiinflammatory activity potential such as α -pinene, β -caryophyllene, camphene and Δ^3 -karen (22-26). However there is no information about the potential use of Cilician fir (*Abies cilicica*) against inflammation, and molecular mechanisms of its potential anti-inflammatory activity are not known.

In this study, to assess anti-inflammatory effects of resin extract obtained from Cilician fir (*Abies cilicica*), two different in vitro inflammation models were used as glucose dependent inflammation and tumor necrosis factor alpha (TNF- α) induced inflammation models. Then its effects on monocyte adhesion, gene expression levels of P-selectin, ICAM-1, VCAM1 and transendothelial migration for the two in vitro models were measured. Also, total flavonoid and total phenolic contents of the extract were determined.

MATERIALS AND METHODS

Plant material

Abies cilicica was collected from the Taurus Mountains (Antalya, Turkey) in 2016. The plant was identified

by Prof. Dr. E. Akalin and deposited in the Herbarium of the Faculty of Pharmacy at Istanbul University.

Extraction of Abies cilicica

Resin (Mezda gum) extract of the *Abies cilicica* plant was used. The rosin was left to dry at room temperature, and extracted by using methanol. The methanol extracts were centrifuged at 600 xg for 30 minutes at 4 °C. After centrifugation, the supernatant was transferred to another well and filtered using an injection filter, and kept at 80 °C.

Mammalian Cell Culture

ECV304 human vascular endothelial cells and U937 cells were used for the inflammation models. Cells were grown in DMEM (for ECV304 cells) and RPMI (for U937 cells) mediums containing Penicillin/Streptomycin, L-glutamine and 10% Fetal Bovine Serum (FBS).

Cell Viability Assay

MTT (3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl tetrazolium bromide) viability test was performed to determine the appropriate dose range of the extract for ECV304 cells (27). The ECV304 cells were counted and adjusted to 1×10^5 cells per mL. The same number of 90 μ L cells were added to placed in 10 μ L wells at 6 different concentrations of the extract; 5000, 500, 100, 10, 1 μ g / mL. As control, 10 μ l medium was added instead of the extract. Plates were incubated in a 37 °C incubator containing 5% Co₂ for 2 days in a humidified atmosphere followed by addition of 10 μ L MTT (5mg/mL) to each well and incubated for 4 hours in an incubator. After incubation, 80 μ L of the supernatant in the wells discarded and 100 μ L of 50% sodium dodecyl sulfate dissolved in isopropyl alcohol (SDS, pH:5.5) was added. The color was measured at 570 nm relative to the reference wavelength of 630 nm on an ELISA spectrometer. Cells incubated with the medium alone were used as negative controls.

In vitro TNF- α and Glucose Dependent Inflammation Models

Two different models of inflammation were generated by stimulating ECV304 cells with glucose and TNF- α . For both in vitro inflammation models, first ECV304 human endothelial cells were incubated with *Abies cilicica* extract 20 μ g / mL for 24 hours. Then

inflammation was induced in ECV304 cells with 30 mM glucose (24 hours) or TNF- α at 5 ng/mL (6 hours) (28,29). In each control group, the same stimulations were made without the extract incubation.

Monocyte Adhesion Experiments

The monocyte adhesion experiments were performed according to the protocol of Choi et al. using ECV304 human endothelial cells and U937 monocyte cells (30). First, ECV304 cells were incubated with the extract at the 20 $\mu\text{g} / \text{mL}$ determined relative to the cytotoxicity result for 24 hours, and induced with 30 mM glucose (24 hours) or TNF- α at 5 ng/mL (6 hours). After treatment of the cells with glucose or TNF- α , U937 monocyte cells were added on ECV304 cells and incubated for 20 minutes at 37 °C. Unbound cells were removed by washing 3 times with serum-free DMEM medium. Connected cells were counted. As control groups: 1) without the extract incubation for two inflammation models and 2) added monocyte and the extract without forming the inflammation models were used.

Determination of Expression Levels of Adhesion Molecules Gene

ECV304 cells were incubated with 20 $\mu\text{g} / \text{mL}$ *Abies cilicica* extracts and then qPCR method was used to investigate changes in the expression levels of P-selectin, ICAM-1, VCAM1 genes in the inflammation models. Real time PCR was performed by using Roche LightCycler@FastStart DNA Master SYBR Green I kit. Manufacturer's protocol was used for RT-PCR test. Reverse primer 5'-AAGGTGAGAGTTGCATTCTCAGAAAG-3' and forward primer 5'-TTACACATTGATGAAATGGATTCTGT-3' were used to determine changes of VCAM-1 gene expression levels. For ICAM-1 gene, the forward primer was 5'-GCAGCCGCAGT-CATAA-3', and reverse primer was 5'-CCCAATAGGCAGCAAGT-3'. For P-selectin gene, forward primer was 5'-GGTCAACTACCGTGCCAACCT-3', and reverse primer was 5'-GACTTACCACCGCTCCA-3'.

Monocyte Migration Assay

The monocyte migration assay was performed according to the protocol of Hsu et al. (29). For monocyte transendothelial migration experiments, a transwell plate containing 24 well-filtered polycarbonate membranes were used in both in vitro inflammation models. ECV304 cells were seeded on top of the trans-well filter and inflammation patterns were established. U937

monocyte cells were then added to the top and incubated for 4 hours at 37 °C in the incubator. At the end of the incubation, monocyte cells passing to the lower part were collected and counted. As control groups: 1) without the extract incubation for two inflammation models and 3) added monocyte and extract without stimulation of glucose or TNF- α were used.

Determination of total flavonoid and phenolic contents

To assess total flavonoid content of the extract, quercetin was used. Stock solution of quercetin was prepared as 0.1 mg/mL and then it was diluted as 6.25, 12.5, 25, 50, 80, 100 $\mu\text{g}/\text{mL}$. The extract (0.5 mL) incubated with 1.5 mL methanol, 0.1 mL 1M potassium acetate, 0.1 mL 10% of aluminum chloride and 2.8 mL distilled water 30 minutes at room temperature. The final color was measured at 415 nm on an UV-vis spectrometer (Thermo Scientific, USA) and total flavonoid content calculated as quercetin equivalents from a calibration curve (31).

In order to measure total phenolic content of the extract, gallic acid was used as the standard. Stock solution of gallic acid as 0.1 mg/mL and then it was diluted as 6.25, 12.5, 25, 50, 80, 100 $\mu\text{g}/\text{mL}$. The extract (0.5 mL) incubated with 2.5 mL 10% of Folin-Ciocalteu and 2.5 mL of sodium carbonate (75g/L) at 45°C for 45 minutes. Then, it was measured at 765 nm on a microplate reader using a UV-vis spectrophotometer and total phenolic content was calculated as the gallic acid standard equivalents from a calibration curve (32).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) program was used for statistical analysis of results. Statistically significant differences were analyzed using Student's t-test. Analyzes were statistically significant with statistical significance of $p < 0.05$.

RESULTS

To determine the optimal dose of cone's rosin extract of *Abies cilicica* against ECV304 cells, MTT assay was applied. The extract showed no toxic effect at concentrations of 50, 10, 5 and 1 $\mu\text{g}/\text{mL}$ while 500 $\mu\text{g}/\text{mL}$ concentration of the extract showed cytotoxic effect (Figure 1).

Monocyte (U937) cells adhering to ECV304 cells in each of the inflammation models were counted. It was found that resin extract increased monocyte adhesion

in TNF- α induced inflammation model with an 100% increase when compared to only TNF- α incubated ECV304 cells. These results suggested that the extract has a triggering effects on TNF- α induced inflammation (Figure 2). On the other hand, glucose stimulation of ECV304 cells caused an increase in monocyte adhesion relative to controls. However, the extract did not change number of adherent U937 cells in glucose dependent inflammation model (Figure 3).

To understand effects of resin extract on diapedesis, a trans-well plate containing a filtered polycarbonate membrane was used in the monocyte migration assay. Monocyte (U937) cells were added to the ECV304 cells seeded in the upper compartment, and counted U937 cells that passed through lower compartment. Monocyte migration assay results were shown in Figure 2 and 3.

Administration of TNF α on ECV304 cells lead to an increase in migration of leukocytes across ECV304 cells. After incubation with the extract of ECV304 cells, number of migrated U937 cells was similar with only TNF α incubation experiment results (Figure 2) However, the extract significantly decreased monocyte migration in glucose dependent inflammation model (Figure 3).

To address the underlying mechanism of monocyte adhesion, we measured whether the extract treatment of ECV304 cells diminishes expression of the adhesion molecules P-selectin, VCAM-1 and ICAM-1 in ECV304 cells stimulated with TNF- α or glucose (Figure 4 and 5). According to our results, both TNF- α and glucose stimulation of ECV304 cells decreased ICAM-1 gene expression levels. We did not find an appreciable effect on VCAM-1 gene expression of ECV304 cells for two in vitro experimental inflammation models. On the other hand, while treatment of ECV304 cells with the extract resulted in a 8-fold increase of P-selectin gene expression, after TNF- α stimulation of ECV304 cells, the extract did not change expression levels of P-selectin gene. However, the extract significantly increased P-selectin gene expression in glucose dependent inflammation model.

Also flavonoid and phenolic contents of the extract were measured. According to the standard of curve of quercetin graph, flavonoid content the extract was found 112 \pm 4.22 mg/g (Figure 6 and Table 1). On the other hand, based on standard of curve of gallic acid graph (data not shown) its phenolic content was determined as 559 \pm 5.6 mg/g (Figure 7 and Table 2).

DISCUSSION

In order to understand cytotoxic effect of the extract was investigated in ECV304 cells by means of the colorimetric MTT assay. The extract showed proliferative effect

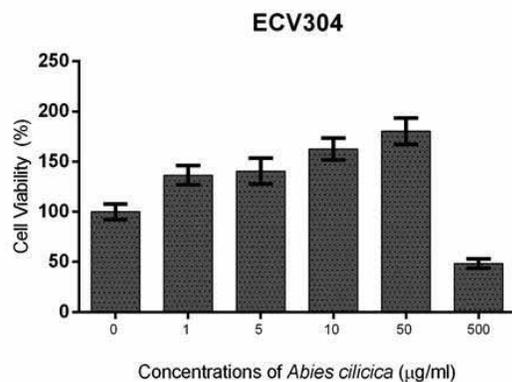


Figure 1. The cytotoxic effect of the resin extract of *Abies cilicica* used in the study against ECV304 cells was demonstrated. The graph shows the level of cell viability according to the concentration of the extract. The control group was accepted as 100%, and cell viability was shown at baseline, at 500, 50, 10, 5 and 1 µg/mL, respectively. The results are shown as mean adhesion \pm SEM.

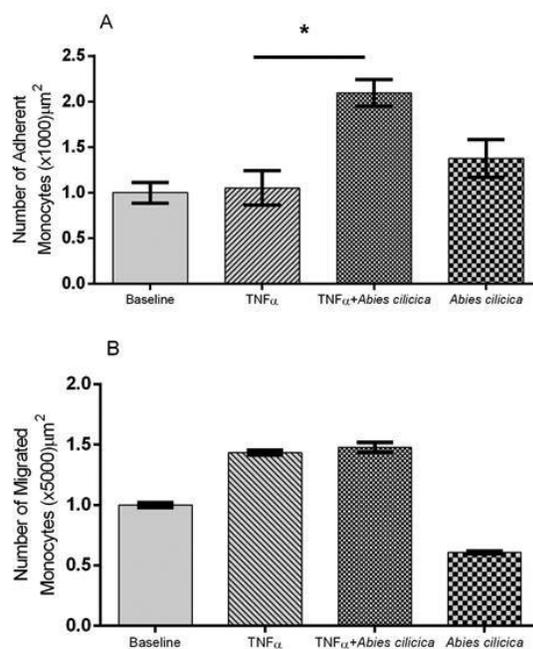


Figure 2. Adhesion and migration of monocytes in TNF- α induced inflammation model in the absence and presence of the resin extract of *A. cilicica* were shown in this figure. The results are shown as mean adhesion \pm SEM. * p < 0.05 is considered significant

on the cells in different concentrations except the highest concentration 500 µg/mL. Considering the results, it can be said that it is not cytotoxic for ECV304 cells.

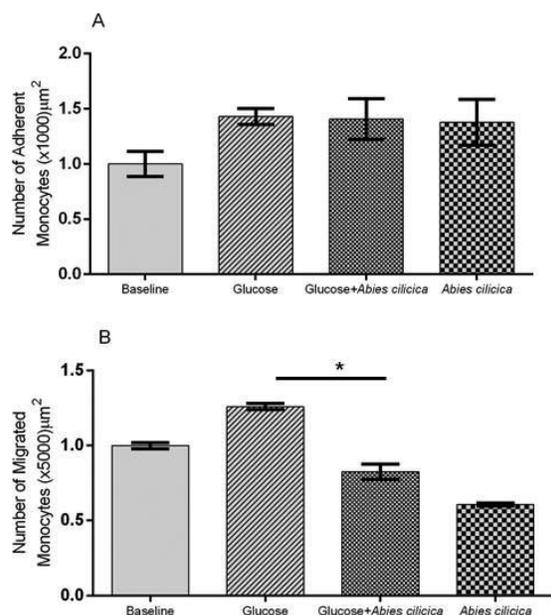


Figure 3. Adhesion and migration of monocytes in glucose dependent inflammation model in the absence and presence of the resin extract of *A. cilicica* were shown in this figure. The results are shown as mean adhesion \pm SEM.

Monocyte adhesion tests showed that the extract increased 100% inflammatory effect of TNF- α induced inflammation (Figure 2). On the other hand, it did not change number of adherent U937 cells in glucose dependent inflammation model (Figure 3). These results indicate that cone's extract may have a selective trigger effect on monocyte adhesion in TNF- α mediated inflammation.

ICAM-1 and VCAM-1 are important in the attachment of leukocytes to endothelial cells. Also, P-selectin is an adhesion molecule stored in endothelial cells, acts an important role in early inflammation. Reactive oxygen species (ROS), mitogen-activated protein kinases (MAPKs), and transcription factors play a role in ICAM-1 expression. Also it was suggested that pro-inflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1 β (IL-1 β), and tumor necrosis factor- α (TNF- α) induce upregulation of ICAM-1 expression (1). In our both experimental inflammation model, ICAM-1 expression significantly decreased.

Type 2 diabetes mellitus (T2D) is a risk factor for atherosclerosis and increases incidence of cardiovascular morbidity and mortality. It was demonstrated that ICAM-1, VCAM-1, E-selectin, and P-selectin are elevated in the early stage of T2D. However, the underlying mechanisms of vascular abnormalities in T2D are not clear (2).

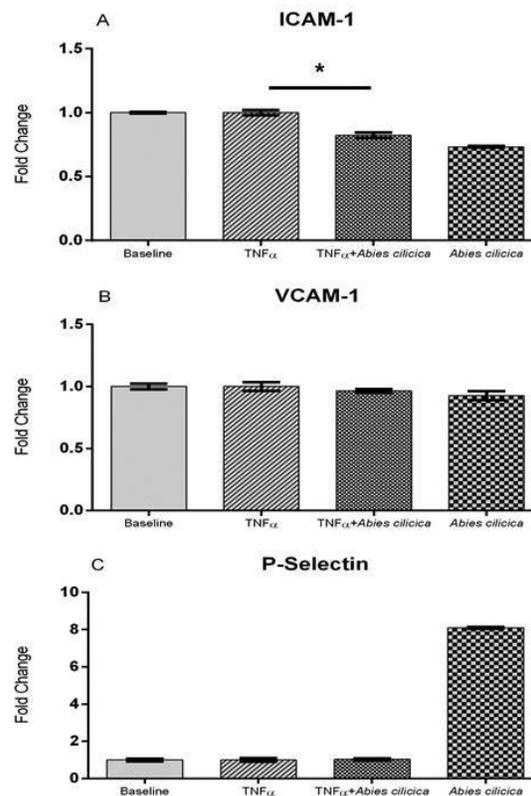


Figure 4. Changes in ICAM-1, VCAM-1 and P-Selectin gene expressions in TNF- α induced inflammation model in the absence and presence of the extract. The results are shown as mean adhesion \pm SEM. * $p < 0.05$ is considered significant.

Although the extract has trigger effect on monocyte adhesion, it did not change migration of leukocytes across ECV304 cells after administration of TNF α on ECV304 cells. The number of migrated U937 cells was similar with only TNF α incubation experiment results (Figure 2) However, it significantly decreased monocyte migration in glucose dependent inflammation model (Figure 3).

According to our results, monocyte adhesion and migration tests show that resin extract of *A. cilicica* has more protective effect against glucose-dependent inflammation. It can be suggested that different signaling pathways in different inflammation models might be the reason for its specific reaction against glucose-dependent inflammation model in vitro.

Flavonoids have many properties such as anti-inflammatory, enzyme inhibition, anti-microbial, anti-allergic vascular and cytotoxic, anti-tumor and anti-oxidant (33). Also they are effective anti-inflammatory compounds for alternative natural treatments (34). Amounts of flavonoid and phenolic content of plants are associated with their antioxidant capacities (31). Previously,

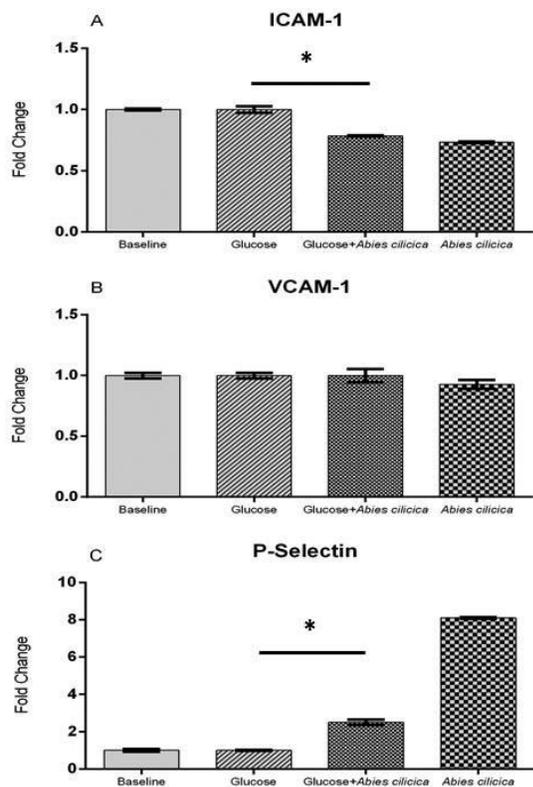


Figure 5. Changes in ICAM-1, VCAM-1 and P-Selectin gene expressions in glucose dependent inflammation model in the absence and presence of the extract. The results are shown as mean adhesion \pm SEM. *p < 0.05 is considered significant.

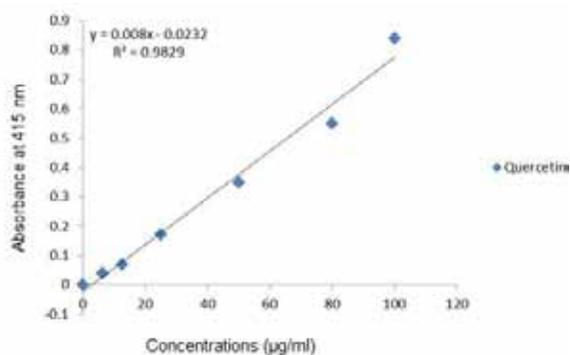


Figure 6. Standard curve of quercetin (0.1 mg/ml stock solution)

several medical plants were shown to have anti-inflammatory action several diseases including diabetes. For example, *Anti-inflammatory effects of Dioscoreophyllum cumminsii* leaves were demonstrated (35). *Bauhinia*

Table 1. Absorption levels of quercetin concentrations at 415 nm.

Concentration of quercetin ($\mu\text{g/ml}$)	Absorption at $\lambda_{\text{max}} = 415 \text{ nm}$
0	0
6.25	0.04
12.5	0.07
25	0.17
50	0.35
80	0.55
100	0.84

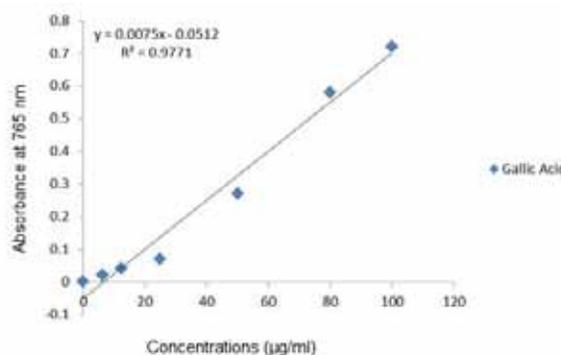


Figure 7. Standard curve of gallic acid (0.1 mg/ml stock solution)

Table 2. Absorption levels of gallic acid concentrations at 765 nm

Concentration of gallic acid ($\mu\text{g/ml}$)	Absorption at $\lambda_{\text{max}} = 765 \text{ nm}$
0	0
6.25	0.02
12.5	0.04
25	0.07
50	0.27
80	0.58
100	0.72

holophylla (Bong.) Steud. (Fabaceae) which is a medicinal plant is used to reduce inflammation (36). Protective effect of the extract of *Portulaca oleracea* against inflammation in diabetes was shown (37). Tartary buck-

wheat bran extract was demonstrated having beneficial effects in endothelial inflammation (38). *Pterocarpus marsupium* ameliorates the inflammation in diabetes (39). *Origanum vulgare* L. ssp. *hirtum* (Greek oregano) has anti-inflammatory compounds having beneficial effects against diabetes (40). Similarly, our results showed that a medical plant, cone's rosin extract of *A. cilicica* containing flavonoid and phenolic compounds has anti-inflammatory effects against the glucose-dependent inflammation model. Phenolic antioxidant capacity of the extract are higher than its flavonoid contents. Oxidative stress can play a role in the pathogenesis of diabetic complications (41). Yin et al. found that phenolic antioxidants such as ellagic acid and kaempferol can prevent oxidative stress in vivo (42).

According to results of various studies including GC-MS analysis of *Abies cilicica* extract, it was shown that mainly essential oils of the extract were α -pinene, β -caryophyllene, camphene and Δ^3 -karene (43). Anti-inflammatory effect of camphene was demonstrated (43,44). On the other hand, it was suggested that β -caryophyllene decreased levels proinflammatory cytokines and showed anti-inflammatory effect in the experimental autoimmune encephalomyelitis model (45). Also, Δ^3 -karene and α -pinene anti-inflammatory and anti-cancer effects have been shown (46).

There are various essential oils in *Abies cilicica* extract. It is known that antagonistic or synergistic effects arising among essential oils can modify the response against inflammation. Therefore isolation of essential oils from the extract and their various combinations can contribute to discover new anti-inflammatory agents. Although its contents showed different effects on the inflammation models, it can decrease ICAM-1 gene expression levels for both the inflammation models. However, signal transduction differences of TNF α and glucose on ECV304 cells may change anti-inflammatory effects of the extract on these cells.

We explored the anti-inflammatory effects of resin extract of Cilician fir (locally called Mezda gum) extract against glucose-dependent inflammation. This study provides the first evidence that the extract inhibits glucose dependent inflammation. Although further effort is desired to define these effects of the extract, it could prevent monocyte adhesion to endothelial cells and transendothelial migration. This extract may serve as an anti-inflammatory agent in the treatment of chronic inflammation caused by diabetes.

ACKNOWLEDGEMENTS

This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK)

2209-A (Project No: 1919B011502641). The authors have no conflict of interest.

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