

Apilarnil protects the LPS induced endotoxemic heart

Apilarnil LPS ile indüklenen endotoksemik kalbi korur

Aslı OKAN¹ (ID), Emin KAYMAK¹ (ID), Arda Kaan ÜNER² (ID), Sibel SİLİCİ³ (ID), Züleyha DOĞANYİĞİT¹ (ID)

ABSTRACT

Objective: Endotoxemia is a serious complication that is featured by hypotension, weak tissue perfusion and multiple organ failure that is among the top factors that lead to mortality in hospitalized patients. Apilarnil (drone bee brood) is a honeybee product that has biologically active characteristics. Apilarnil contains 25 - 35% dry matter, 9 - 12% protein, 6 - 10% carbohydrates, 5 - 8 lipids, 3% ash, and other unidentified substances. Additionally, its chemical composition includes vitamins (vitamin A, beta carotene, B1, B6, PP, and choline), minerals (calcium, phosphorus, sodium, zinc, manganese, iron, copper, and potassium), and essential amino acids that cannot be synthesized by humans or other animal organisms. Endotoxemia-induced cardiotoxicity is reported in the recent evidence. In this study, it was aimed to determine whether apilarnil, a bee product, has a protective effect on heart tissue in the case of endotoxic shock, which is one of the major causes of intensive care units, by histopathological and immunohistochemical evaluation of TNF- α and BNP in the heart tissue.

Methods: 32 *Sprague dawley* male rats were randomly divided into four equal groups as control, apilarnil administered group (0.8 g/kg), lipopolysaccharide (LPS)

ÖZET

Amaç: Endotoksemi, hastanede yatan hastalarda mortaliteye neden olan faktörlerin başında gelen hipotansiyon, zayıf doku perfüzyonu ve çoklu organ yetmezliği ile karakterize ciddi bir komplikasyondur. Apilarnil (drone arı kuluçkası), biyolojik olarak aktif özelliklere sahip bir bal arısı ürünüdür. Apilarnil %25-35 kuru madde, %9 - 12 protein, %6 - 10 karbonhidrat, 5 - 8 lipid, %3 kül ve diğer tanımlanamayan maddeler içerir. Ek olarak, kimyasal bileşimi vitaminleri (A vitamini, beta karoten, B1, B6, PP ve kolin), mineralleri (kalsiyum, fosfor, sodyum, çinko, manganez, demir, bakır ve potasyum) ve insanlar veya diğer hayvan organizmaları tarafından sentezlenemeyen esansiyel amino asitleri içerir. Son kanıtlarda endotoksemimin neden olduğu kardiyotoksikite rapor edilmiştir. Bu çalışmada, bir arı ürünü olan apilarnilin, yoğun bakım ünitelerinin önemli nedenlerinden biri olan endotoksik şok durumunda kalp dokusunu koruyucu etkisinin olup olmadığının kalp dokusunda histopatolojik ve TNF- α ve BNP immunoreaktivitesindeki değişimler ile araştırılması amaçlanmıştır.

Yöntem: 32 adet *Sprague dawley* erkek sıçan rastgele kontrol, apilarnil uygulanan grup (0.8 g/kg), lipopolisakkarit (LPS) grubu ve apilarnil ile birlikte LPS

¹Yozgat Bozok University, Faculty of Medicine, Department of Histology and Embryology, Yozgat

²Yozgat Bozok University, Faculty of Medicine, Yozgat

³Erciyes University, Faculty of Agriculture, Department of Agricultural Biotechnology, Nutral Therapy Co. Erciyes Technopark, Kayseri



İletişim / Corresponding Author : Züleyha DOĞANYİĞİT

Yozgat Bozok Üniversitesi Tıp Fakültesi, Yozgat - Türkiye

E-posta / E-mail : zuleyha.doganyigit@gmail.com

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group, and apilarnil together with LPS administered group. Immunohistochemical evaluation was performed to determine the differences in the expression of TNF- α and BNP in the heart tissue.

Results: Edema, hemorrhage, and infiltration was observed in the LPS group compared to the control group and groups receiving apilarnil. It was observed that this damage decreased significantly in the group treated with LPS and apilarnil. Tumor necrosis factor-alpha (TNF- α) and brain natriuretic peptide (BNP) expressions were significantly increased in the LPS group, and co-administration of LPS and apilarnil suppressed these increased expression levels.

Conclusion: Our data indicate that the protective effects of apilarnil may have a therapeutic effect on heart damage caused by LPS by the anti-inflammatory and antioxidant properties of the components found in apilarnil.

Key Words: LPS, Apilarnil, BNP, TNF- α , bee product

verilen grup olmak üzere dört eşit gruba ayrıldı. Kalp dokusunda TNF- α ve BNP ekspresyonundaki farklılıkları belirlemek için immünohistokimyasal değerlendirme yapıldı.

Bulgular: Kontrol grubu ve apilarnil alan gruplara göre LPS grubunda ödem, kanama ve infiltrasyon gözlemlendi. LPS ve apilarnil ile tedavi edilen grupta bu hasarın önemli ölçüde azaldığı gözlemlendi. LPS grubunda tümör nekroz faktör-alfa (TNF- α) ve beyin natriüretik peptit (BNP) ekspresyonları önemli ölçüde arttı ve LPS ve apilarnilin birlikte uygulanması bu artan ekspresyon seviyelerini baskıladı.

Sonuç: Verilerimiz, apilarnilin koruyucu etkilerinin, apilarnilin içerdiği bileşenlerin antiinflamatuvar ve antioksidan özellikleri sayesinde LPS'nin neden olduğu kalp hasarı üzerinde terapötik bir etkiye sahip olabileceğini göstermektedir.

Anahtar Kelimeler: LPS, Apilarnil, BNP, TNF- α , arı ürünü

INTRODUCTION

Endotoxemia is a serious complication that is featured by hypotension, weak tissue perfusion and multiple organ failure which is among the top factors that lead to mortality in hospitalized patients (1). While a lot of strategies have been advanced to understand and treat the pathophysiology of endotoxemia induced by endotoxins, especially LPS (2), it is still one of the most significant problems at intensive care units. Some important events develop in sepsis pathophysiology. The first of these is that monocyte, macrophage, neutrophil, and endothelium cells play an important role in initiating and sustaining response against sepsis (3, 4). Other significant events in sepsis pathophysiology are inflammation and coagulation (5). By the time inflammation starts,

excessive and uncontrolled increase in the formation of mediators such as cytokines, reactive oxygen, and nitrogen species contributes to the pathophysiology of sepsis (6, 7). The endotoxin that is released during the fast growth of the cell or cell destruction is the key molecule that starts the series of events in sepsis/endotoxemia (8). LPS is a glycolipid component of the cell membrane of gram (-) bacteria, and LPS has a harmful effect that leads to septic shock and death on some organs including the heart. Endotoxemia-induced cardiotoxicity is described by overabundant accumulation of reactive oxygen species (ROS), membrane lipid damage, induction of DNA and protein and unsuitable intracellular redox balance (9).

TNF- α plays an important role in the described of cardiovascular diseases and sepsis-induced cardiac function disorders. While it was initially described

as only an LPS - based macrophage product, there is evidence showing that heart myocytes themselves in addition to LPS produce significant amounts of TNF- α corresponding to ischemia (10). Although many natriuretic peptides are secreted from the heart because of dysfunction in the heart, the most sensitive and up to date among these BNP (11). BNP is a hormone that plays a role in some functional changes, particularly volume change and it is a sensitive marker of dysfunction in the heart (12-14). Due to their functions in the pathogenesis of cardiovascular diseases, this study also aimed to immunohistochemically determine the expression levels of TNF- α and BNP in heart tissue.

It is seen in recent years that alternative treatment options in addition to medical treatment have gained increasing significance, especially in the field of human medicine. Apilarnil (drone bee brood) is a honeybee product that has biologically active characteristics. It is obtained by collecting and lyophilizing male bee larvae that are harvested in the 7-day larva stage before the honeycomb cells are closed. It has a homogenous and milk-like consistency, yellowish color and sour taste. Apilarnil contains 25 - 35% dry matter, 9 - 12% protein, 6 - 10% carbohydrates, 5 - 8 lipids, 3% ash and other, unidentified substances (15, 16). Additionally, its chemical composition includes vitamins (vitamin A, beta carotene, B1, B6, PP, and choline), minerals (calcium, phosphorus, sodium, zinc, manganese, iron, copper, and potassium) and essential amino acids that cannot be synthesized by humans or other animal organisms. It was reported that it is a rich source of coenzyme Q10 and contains alpha tocopherol (17). There are various studies on the effects of apilarnil on gastrointestinal diseases, respiratory tract diseases and especially sexual functions (18-21). In this study, we aimed to histologically and immunohistochemically determine whether apilarnil has a protective effect on heart tissue in cases of endotoxemia, which is one of the greatest problems of intensive care units at hospitals.

MATERIAL and METHOD

Chemicals

Lyophilized apilarnil purchased from Nutral Therapy Company (Erciyes University Technopark, Kayseri, Turkey) was used for experiments. LPS (*Escherichia coli* LPS, serotype 0127: B8) was obtained from Sigma Aldrich. The LPS and apilarnil doses to be applied to the rats were determined based on the information in the literature (18, 22, 23). As the number of studies on the biological activity of apilarnil in rats is limited, this study would also be a dose determination study, and the dose range was kept broad in a way to cover the doses proposed in previous studies on other animal groups (18, 22).

Animals and Experimental Protocols

The study used adult male *Sprague dawley* rats that were bred at the DEKAM at Erciyes University. The water and food needs of the rats that were kept in cages were met through the normal course of the day at 21 °C and with a 12-hour light/ dark cycle. The number and breed of the animals to be used in the study and the doses to be applied were determined based on the information in the literature.

For the study, 32 adult male *Sprague dawley* rats were randomly divided into 4 equal groups.

Group 1: (Control group): The group was given only physiological serum (SF) (0.9% NaCl) by 1 mL intraperitoneally,

Group 2: LPS: 30 mg / kg / bw dose 1 mL intraperitoneally,

Group 3: 0.8 g / kg apilarnil 1 mL by oral gavage (every day for 10 days),

Group 4: 0.8 g / kg apilarnil 1 mL by oral gavage (every day for 10 days), LPS (30 mg / kg / bw) on the 10th day 60 minutes after apilarnil administration, six hours after LPS administration, the rats were sacrificed (23), and their heart tissues were collected for histological and immunohistochemical examinations.

Histological Analysis

After the heart tissue that was collected was put into formaldehyde, it was fixated for 2 days in parallel to the size of the tissue, and the tissue was kept under running water overnight following fixation (25). The heart tissues were then embedded in paraffin blocks with the method of routine histological follow up. Samples of 5 μ - thick cross- sections taken from these blocks onto slides were firstly deparaffinized for histochemical staining, and they were stained with hematoxylin & eosin. The tissues were subjected to histochemical analyses under an Olympus BX53 light microscope.

Immunohistochemical Analysis

To determine the differences in the expression of TNF- α and BNP in the heart tissue, marking was performed with the avidin-biotin-peroxidase method (24). Briefly, after deparaffinization and rehydration by passing through gradual alcohol series of 5 μ m cross - sections, 5% citrate buffer (pH: 6.0) was used for regaining antigens. Then the sections were treated with 3% hydrogen peroxide (H₂O₂) to prevent endogenous peroxidase activity. Serum block was applied to prevent non-specific staining. After that, the cross-sections were kept at 4 °C overnight by primary antibodies (TNF- α and BNP separately). After the washing process, biotinylated secondary antibodies, avidin- biotin enzyme and DAB chromogens were applied, respectively. Then

sections were counterstained with Gill hematoxylin, dehydrated with increasing gradual alcohol series, xylol and mounted in entellan. The images that were obtained by a digital camera (DP71 model) under an light microscope (Olympus BX51 model) were analyzed by using the image j software for expression level differences.

Statistical Analysis

SPSS 22 was used for the statistical analyses. For the intergroup comparisons of the obtained expression values, the parametric test one - way ANOVA was utilized, while post - hoc Tukey's test was used for multiple comparisons.

This study was approved by the Erciyes University Animal Experiments Local Ethics Committee (Date:09.05.2018, Number: 18/063).

RESULTS

Histological Analysis

The general structure of the heart tissue was observed by hematoxylin & eosin staining. While a healthy heart tissue was observed in the control group and the groups that were given apilarnil, occasional structural irregularities, hemorrhage and edematous areas were seen in the group that was given LPS. Close to normal heart tissue was observed in the group that were given apilarnil in addition to LPS (Figure 1).

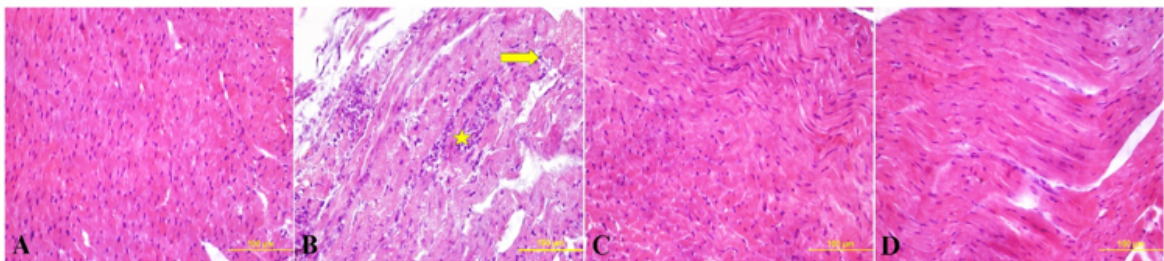


Figure 1. Histological analysis of heart tissues of study groups (H&E). Control group (A), LPS group (B), the group given 0.8 mg/kg dose of Apilarnil (C) and the group given LPS+0.8 mg/kg dose of Apilarnil (D). Magnification rate: x 400.

Immunohistochemical Analysis

Table 1 shows that the TNF- α and BNP immunoreactivity results increased in the LPS group significantly in comparison to the control group ($p=0.001$). While these expression levels in the group where the only apilarnil was applied were like

those in the control group, similar results were also observed in the groups given apilarnil in addition to LPS (Figure 2, 3 and 4). It is seen that the apilarnil applied alongside LPS reduced the damage induced by LPS.

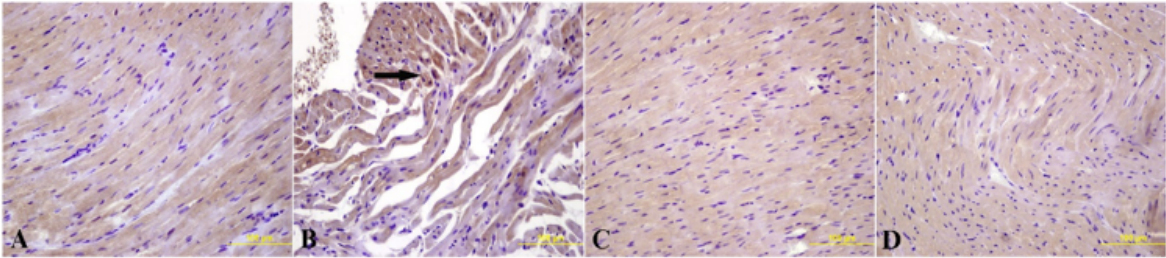


Figure 2. Immunohistochemical analysis of TNF- α at heart tissues of study groups. TNF- α images of the Control group (A), LPS group (B), the group given 0.8 mg/kg dose of Apilarnil (C) and the group given LPS+0.8 mg/kg dose of Apilarnil (D). The arrow shows the stained areas. Magnification rate: x 400.

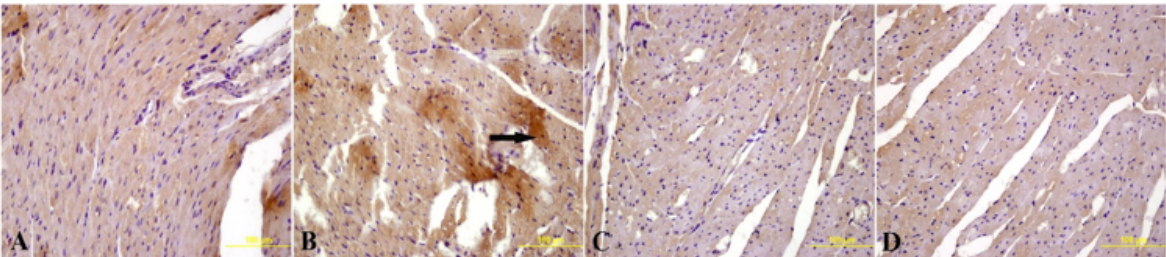


Figure 3. Immunohistochemical analysis of BNP expression at heart tissues of study groups. Control group (A), LPS group (B), the group given 0.8 mg/kg dose of Apilarnil (C) and the group given LPS+0.8 mg/kg dose of Apilarnil (D). The arrow shows the stained areas. Magnification rate: x 400.

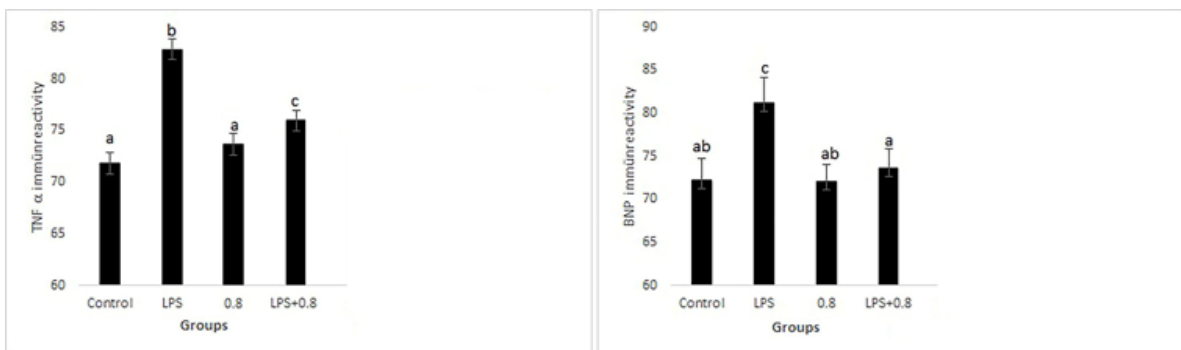


Figure 4. TNF- α and BNP immunoreactivity data shown on histogram graph are expressed as mean \pm SEM. The data are expressed as mean \pm standard deviation. $p < 0.05$ was accepted as statistically significant. There was no significant difference among the groups indicated with the same letters (a- b- c).

Table 1. Immunoreactivity results of TNF- α and BNP

Groups	Control	LPS	0.8mg/kg Apilarnil	LPS+0.8mg/kg Apilarnil	p
TNF- α	71.82 \pm 1.48 ^a	82.81 \pm 4.28 ^b	73.69 \pm 2.68 ^a	76.00 \pm 5.10 ^c	0.001
BNP	72.24 \pm 2.54 ^{ab}	81.22 \pm 2.91 ^c	72.15 \pm 1.96 ^{ab}	73.67 \pm 2.27 ^a	0.001

Data are expressed as mean \pm standard deviation. $p < 0.05$ was considered significant. There was no significant difference between groups containing the same letter (a-b-c).

DISCUSSION

Endotoxic shock a syndrome involving multiple organ dysfunction and especially cardiovascular diseases. It should be kept in mind that cardiovascular diseases continue to be the main reasons for morbidity and mortality in the world (25). The pathophysiology of endotoxemia involves the formation of inflammatory mediators such as TNF- α , interleukin-1 (IL-1), IL-2 and IL-6 (26). The cardiovascular changes that are observed during a hyperdynamic septic event are usually characterized by normal to high cardiac output, low systemic vascular resistance, intense hypotension, and deep peripheral vasodilatation (27). The myocardial levels of TNF- α , IL-1 β and IL-6 increased in animals treated with LPS in comparison to controls (28).

Data obtained from scientific studies show that cytokines are mediators of cardiovascular disease (29-31). Endotoxemia is characterized by actuation of the immune system and increased the production of a few cytokines such as TNF- α and c-interferon in the circulation (32). TNF- α is a proinflammatory cytokine that plays a role in sepsis-related myocardial dysfunction (31). It is well-known that TNF- α is an important inducer of myocardial depression during sepsis (28). The proinflammatory cytokines IL-1, IL-6, and TNF were measured in rat serum and myocardial tissue, and it was shown that all these three cytokines significantly increased after administering LPS (33). Asgharzadeh et al. (34) investigated the effects of repeated LPS exposure in male rats on inflammatory markers, oxidative stress balance, and cardiac and

renal fibrosis and found the serum TNF- α and IL-6 concentrations in the group that was given LPS significantly higher in comparison to the control group. Additionally, in the heart tissues of the group treated with LPS, inflammatory cell infiltration, fiber irregularity, edema and broadened blood vessels were observed in the left ventricle. In our previous study, LPS treatment increased TNF- α , IL-6 and IL-1 β in rat liver tissue, while both LPS and apilarnil administration reduced this increase (25). In another study evaluated serum TNF- α and IL-1 β levels and showed that these levels were higher in the animals in the LPS group in comparison to those in the control group (35). Considering the results of our study, in agreement with the results in the literature, histopathological changes such as hemorrhage, edema and structural irregularities were observed in the LPS group, and it was shown that these changes were alleviated by administering apilarnil alongside LPS. Likewise, when the expression of TNF- α in the heart tissue was examined immunohistochemically, while the results in the control group and the groups that were given apilarnil were similar, it was observed that LPS administration increased TNF- α expression significantly, and this increase was significantly suppressed in the group that were given apilarnil in addition to LPS.

As a significant biomarker, BNP concentrations in the serum also increase alongside increased hemodynamic stress or ventricular cavity in heart hypertrophy and heart failure (36-38).

Gao et al. (39) conducted a study on BNP expression in rat myocardial tissue after acute cardiac dysfunction

and the role of BNP in the diagnosis of cardiac dysfunction in forensic medicine and observed that the positive staining degree of BNP increased through the time that passed during cardiac dysfunction. BNP expression had a significant increase based on increased time in cardiac dysfunction by Western blot and real-time PCR. Dong et al. (40) measured serum BNP levels by the ELISA method. In comparison to the control group, the BNP levels in the MI groups were significantly higher. In the results of this study, in agreement with those in the literature, it was observed that the BNP expression levels increased significantly in the group that was given LPS, while these levels were suppressed significantly in the group that were given apilarnil alongside LPS. However, the expression level of BNP in the apilarnil-only group was like the control.

Apilarnil, a bee product that attracts the attention of researchers in recent years, has shown positive effects on reproductive functions (18, 19, 21). It has also been reported to be used successfully against gastrointestinal diseases and respiratory diseases in South Africa (20). The literature review did not reveal any study on the cardioprotective effects of apilarnil, so, our study is the first one in this respect. However, studies on other bee products showed the cardioprotective effects of these products. For example, Malaysian Propolis (MP) was shown to have a cardioprotective activity against isoproterenol-induced oxidative stress by its direct cytotoxic radical cleansing activities. Histopathological analyses revealed edema and infiltration in the heart tissue, and propolis application prevented these negative changes. ROS, such as superoxide anions and hydroxyl radicals are cleansed by the antioxidants found in propolis (41). Alyane et al. (42) proposed as a result of their study that propolis has a positive effect on human health based on the protection of heart tissue in patients who are given doxorubicin and according to the literature on biomedicine.

Shen et al. (43) suggested that using *Schisandra chinensis* bee pollen extract (SCBPE) as a functional

food may provide a scientific basis in preventing MI. It is generally accepted that oxidative stress plays a significant role in the pathogenesis of MI. Excessive production of ROS may affect cell membrane characteristics and lead to oxidative damage in lipids and proteins that may turn them dysfunctional (44). For this reason, treatment with antioxidants may be a powerful strategy in preventing heart damage and myocardial function disorders in acute MI patients (45).

The results of the study by Doganyigit et al. (23) demonstrated that propolis application may have a protective effect against changes in both genomic stability values and methylation profiles, and it minimized the tissue damage caused by an increase in MDA and LPS.

Wang et al. (46) reported that application of ethanol extracts Chinese propolis (EECP) and ethanol extracts poplar buds (EEPB) in mice with endotoxemia alleviated histopathological changes in the lungs and provided significant protective effects by suppressing LPS-stimulated inflammatory cytokines such as IL-6, IL-10, MCP-1, TNF- α , IL-12, and p70. Malekinejad et al. (47) reported that the histopathological and biochemical changes caused by paclitaxel were repaired by application of royal jelly. Paclitaxel histologically led to hemorrhage, congestion, edema, and necrosis in heart tissue, and the application of royal jelly reversed these negative changes. Sugiyama et al. (48), showed that 10H2DA is one of the components of royal jelly with anti-inflammatory effects, and it may be a candidate as a therapeutic drug for inflammatory and autoimmune diseases connected to the production of NF- κ B and IL-6.

As a result of this study, we concluded that apilarnil application showed a cardioprotective effect by reducing tissue damage induced by LPS and suppressing the increase in TNF- α and BNP expressions. We believe that this effect was caused by the anti-inflammatory and antioxidant properties of the components found in apilarnil. However, more detailed studies on this topic are needed.

ETHICS COMMITTEE APPROVAL

* This study was approved by the Erciyes University Animal Experiments Local Ethics Committee (Date:09.05.2018 and No:18/063).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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