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Bee pollen increases hippocampal brain-derived neurotrophic factor and suppresses neuroinflammation in adult rats with chronic immobilization stress

Özlem Saral [a,*], Hüseyin Şahin [b], Sinan Saral [c], Mehmet Alkanat [d], Kerimali Akyıldız [e], Atilla Topçu [f], Adnan Yılmaz [g]

[a] Department of Nutrition and Dietetics, Faculty of Health Science, Recep Tayyip Erdogan University, Rize, Turkey
[b] Espiye Vocational School, Giresun University, Giresun, Turkey
[c] Department of Physiology, Faculty of Medicine, Recep Tayyip Erdogan University, Rize, Turkey
[d] Department of Physiology, Faculty of Medicine, Giresun University, Giresun, Turkey
[e] Department of Medical Services and Techniques, School of Health Care Services Vocational, Recep Tayyip Erdogan University, Rize, Turkey
[f] Department of Medical Pharmacology, Faculty of Medicine, Recep Tayyip Erdogan University, Rize, Turkey
[g] Department of Medical Biochemistry, Faculty of Medicine, Recep Tayyip Erdogan University, Rize, Turkey

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ABSTRACT

Chronic stress is a potential problem associated with anxiety, depression, and cognitive dysfunction. Bee pollen, a powerful antioxidant, has many therapeutic effects. In this study, we aimed to examine the effects of one of the Anatolian bee pollens on depression/anxiety. 24 male Sprague Dawley rats were divided into 3 groups as control, stress, and bee pollen + stress. Bee pollen (200 mg/kg/day) was given to rats exposed to physical stress for 10 days. Open field test (OFT) and forced swimming test (FST) were applied to monitor the behavioral changes of the rats. After behavioral tests, the rats were euthanized. Brain-derived neurotrophic factor (BDNF), interleukin 1 beta (IL-1β), and tumor necrosis factor-alpha (TNF-α) levels were measured by ELISA to evaluate neurological and biochemical changes in rat hippocampal tissue. In addition, malondialdehyde (MDA) and glutathione (GSH) levels in the brain were evaluated. According to the behavioral test results, bee pollen reduced anxiety-like behavior but did not affect depression-like behavior. We also found that bee pollen suppressed neuroinflammation while reducing oxidative stress and lipid peroxidation in hippocampal tissues. Moreover, bee pollen significantly increased the level of BDNF in the hippocampus. In conclusion, bee pollen reduced oxidative damage and neuroinflammation caused by immobilization stress in rat brain tissue. Therefore, we suggest that bee pollen may be an effective natural compound in alleviating the negative effects caused by immobilization stress.

1. Introduction

During daily life, modern humans society is simultaneously exposed to many types of stressors such as social, chemical, psychological, and physiological stress [1]. Stress can cause psychiatric disorders such as depression and anxiety, and cognitive impairment [2]. Psychotherapy and drug therapy are combined in the treatment of anxiety or depression. Antidepressants are used as drug therapy, and antidepressants have side effects such as emotional blunting, gastrointestinal problems, insomnia, sexual dysfunction, and suicidal ideation [3]. Therefore, researchers have recently focused on the potential of foods and natural products to prevent stress-related health problems [1].

Bee products known as a member of these natural products have become popular thanks to their potential therapeutic effects in metabolic disorders [4]. Among bee products, besides honey, bee pollen is also a highly preferred product and containing substances that are essential for life. Moreover, bee pollen has been used for centuries in traditional medicine because of its potential nutritional and medical applications. Bee pollen is known as a rich source of protein; actually, its total composition can vary due to its botanical and geographic origin. Besides its current proteins, it also contains carbohydrates, lipids, vitamins, minerals, and phenolic compounds which exhibit various

* Corresponding author.
E-mail address: kimya061@hotmail.com (Ö. Saral).

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biological activities [5–8]. There are some evidence studies are related to biological activities of bee pollen such as anti-inflammatory [9], antioxidant [5,10] and antimicrobial [6] in the literature. As previously reported in studies that are performed *in vivo*, bee pollen acts as a significant neuroprotective agent [8,11–13]. For example in some of those studies related to neurological disorders, it was claimed that bee pollen reduced the level of anxiety [13], and heals cognitive impairment [12]. Also, it has also been reported that bee pollen prevents neurotoxicity caused by heavy metal [8,11] and improves oxidative damage in rodents with autistic properties [14]. The therapeutic and preventing oxidative damage effect of bee pollen is thought to be due to the various phenolic compounds it contains [8,11,14–15].

Immobilization stress is one of the experimental animal models used to evaluate the neuropsychological effects of stress and anti-stress properties of therapeutic agents in rodents [16]. To the best of our knowledge, no study has so far reported on the relationship between immobilization stress and the therapeutic effect of bee pollen. For this reason, we aimed to examine the therapeutic effects of bee pollen, a powerful antioxidant, on depression/anxiety behaviors related to immobilization stress-induced in rats.

2. Material and methods

2.1. Animals and experimental protocol

All experimental protocols were approved by the Local Ethics Committee of Recep Tayyip Erdogan University in Rize, Turkey (2020/23). Adult male 24 Sprague Dawley rats, weighing 300–350 g, divided into three groups (n = 8) as the I) control, II) stress and III) stress + bee pollen (Stress + BP) group. The effective dose of bee pollen used in the present study (200 mg/kg/day) was selected from a previous study [14]. In addition, the immobilization stress protocol has been modified from a previous study [2]. Briefly; the restrictive apparatus used in the experiment was made of a funnel-shaped transparent plexiglass material with dimensions of 6.5 × 6.5 × 23 cm. There are holes in the upper and head area of the apparatus that provide ventilation. The rats were subjected to immobilization stress for 2 h a day (10 days/2 h) for ten consecutive days. Immobilization stress was carried out every day between 14:00 and 16:00 h. During stress, all groups were deprived of food. After the final immobilization stress protocol, OFT and FST were performed.

2.2. Open field test (OFT)

OFT is used to evaluate anxiety-like behavior in rodents [16]. The apparatus used for OFT consists of a square-shaped plexiglass box (90 × 90 × 40 cm) that has a dark grey floor and interior walls. The central area and edge regions were determined by dividing the test area into 10 cm small squares on the software screen. Data were recorded with the behavior monitoring software program ANY maze (ANY maze 5.2, Dublin/Ireland). The rats were placed in the center of the arena respectively and their behaviors were recorded for 300 s. Rats were tested only once. The tests were carried out every day at the same times (14:00–17:00). The platform was cleaned with 70% ethanol solution so that the behavior of each rat was not affected by the residues of the previous animals such as smell and urine. The total distance travelled by the rats (m), average speed (m/s), total immobilization time (s), time spent in the center (s) and the number of access to the center were scored using the software program. Grooming and rearing behavior were scored manually.

2.3. Forced swimming tests (FST)

FST is a commonly used test to evaluate depression-like behavior in rodents [17]. The FST includes the evaluation of rats' passive sedentary behavior and active movements such as swimming and climbing while swimming in a water-filled cylinder with no escape. The rat typically exhibits struggle behavior to escape, but after a while naturally remains immobile by making small movements to keep his head above the water, reflecting a state of behavioral helplessness. The apparatus used in the experiment was made of plexiglass material in standard dimensions (30 cm in diameter, 60 cm in height). On the 12th day of the experiment, the rats were placed in a cylinder filled with 40 cm of water (27 ± 1° C) for 5 min, respectively. Swimming, climbing and immobility behavior scores were recorded with ANY maze 5.2 behaviour software monitoring program.

2.4. Biochemical analysis

One day after behavioral assessment, rats anesthetized with ketamine (50 mg/kg, i.p)-xylazine (5 mg/kg, i.p). The Hippocampus of the brains was removed and weighed. Phosphate buffer (pH 7.4) was added to 2 times the brain tissue weight. It was homogenized with a homogenizer for 30 Hertz/5 min. Finally, it was centrifuged at 3000xg for 15 min and the supernatants obtained were used for biochemical analysis [18]. Brain tissues of BDNF (Mybiosource BDNF kit, catalog no: MBS9139053), TNF-α (Mybiosource TNF-α kit, catalog no: MBS175904) and IL-1β (Mybiosource IL-1β kit, catalog no: MBS2702038) levels were determined in accordance with the instructions of a commercial rat-specific ELISA kit.

Tissue malondialdehyde (MDA) level was performed according to the double heating method of Draper and Hadley [19]. The method is based on its reaction with TBA at 94 °C to form a pink complex with an absorption maximum at 532–535 nm. The results are expressed as nmol/g tissue. Glutathione (GSH) levels were performed using to Ellman method in brain tissue samples [20]. The method is based on the spectrophotometric reading of the yellow complex formed by the reaction of the free sulfhydryl group with Ellman's reagent at 412 nm.

2.5. Chemicals and bee pollen samples

The chemicals used were of analytical purity. Gallic acid, Na_2CO_3 (Sodium carbonate), Folin-Ciocalteu's reagent $FeSO_4{\cdot}7H_2O$ (Iron (II) sulfate heptahydrate), TPTZ (2,4,6-tripyridyl-s-triazine), and methanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Twelve dried Anatolian bee pollen samples collected from different locations of Turkey were obtained from a manufacturing company (The Bee&You brand (Bee'O®) (SBS Scientific Bio Solutions Inc., Istanbul, Turkey) in Istanbul, Turkey. The botanic origin of Anatolian bee pollen samples was recorded according to the declaration of the manufacturing company and the beekeepers, thus the classification was performed as multi-floral without performing microscopic analysis. The supplying info in terms of location is presented along with the current sample code in Table 3.

2.6. Preparation of bee pollen samples for in vitro analyses

In vitro analyses of the bee pollen samples were evaluated in two categories as the determining of antioxidant level and the investigation phenolic profile of the selected sample having the highest antioxidant level. Moreover, this selected sample also was used for the animal application. Approximately, 10 g of each Anatolian bee pollen samples was weighed and dissolved in 50 mL methanol. To increase the degree of homogeneity in extraction, the samples were stirred with a magnetic stirrer (Daihan Scientific Co., Ltd., Korea) for 24 h at room temperature. After stirring, the suspensions were filtered to remove possible particles, and the final extraction volume of the samples was adjusted with methanol. All filtrates adjusted their final volume was kept at 4 °C until the calculation of their antioxidant capacity. According to the antioxidant examination, Kocaeli bee pollen sample **(P5)** due to having the highest data was prepared for the HPLC-UV analysis to reveal it's current phenolic profile. For this application, the selective extraction procedure was preferred previously defined by Akyuz et al. [21] in detail. The current methanolic extract of **P5** was evaporated at 40 °C in a rotary

evaporator (Heidolph, Schwabach, Germany) until methanol dried. After residue dissolving with distilled water, the liquid–liquid extraction procedure was performed with diethyl ether and ethyl acetate consecutively. All organic fractions were collected in the same flask and evaporated in a rotary evaporator under the same conditions. The final residue was dissolved in chromatographic grade methanol for phenolic evaluation with HPLC.

2.7. Determination of total phenolic content (TPC)

The using methodology of TPC known as Folin-Ciocalteu assay is based on the calculation of the change in the spectrophotometric absorbance at 760 nm [22]. For this application, 680 μL distilled water, 20 μL methanolic samples, 400 μL of 0.5 N the reagent of Folin-Ciocalteu, and 400 μL of 10% Na_2CO_3 were added to a test tube and incubated for 2 h at room temperature. Absorbance was measured against a blank at 760 nm. The standard calibration curve was plotted using gallic acid (1–0.031 mg/mL $R^2 = 0.9991$). The value of TPC was expressed as mg Gallic equivalents per g sample (mg GAE/g).

2.8. Ferric reducing antioxidant power (FRAP)

FRAP activity is mentioned as the reduction of ferric- tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) by the literature [23] was related to the current antioxidants of analyzed pollen samples. Briefly, FRAP reagent was prepared by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL of 10 mM TPTZ solution in 40 mM HCl, and 2.5 mL of 20 mM $FeCl_3{\cdot}6H_2O$ solution. Then, 100 μL methanolic bee pollen sample and 3 mL freshly prepared FRAP reagent were pipetted in a test tube. After incubation for 4 min at 37 °C, absorbance was recorded at 595 nm against the blank reagent. The standard calibration chart was prepared using $FeSO_4{\cdot}7H_2O$ standard from 1000 μM to 31.25 μM ($R^2 = 0.9995$). FRAP values were expressed as μM $FeSO_4{\cdot}7H_2O$ equivalent of g sample.

2.9. Determination of phenolic compounds by HPLC

For the evaluation of the phenolic compounds, the chromatographic procedure previously mentioned in a study was followed Sahin et al. [24]. Eighteen standards (gallic acid, protocatechuic acid, protocatechuic aldehyde, *p*-hydroxybenzoic acid, catechin, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, epicatechin, vanillin, *p*-coumaric acid, syringaldehyde, rutin, ferulic acid, benzoic acid, rosmarinic acid, and quercetin) were defined in terms of qualitative and quantitative by considering their retention time and UV spectra in an HPLC device (Thermo Scientific Dionex Ultimate™ 3000 system, Thermo Scientific, Germany). For each chromatographic run completed in 45 min, the binary gradient mobile phase program which was consisted of 2% (v/v) acetic acid in water and 50% (v/v) acetonitrile containing 0.5% (v/v) acetic acid in water was used with a C_{18} column (250 mm × 4.6 mm × 5 μm; Thermo Scientific™ Hypersil™ ODS C_{18} HPLC, Thermo Scientific, Waltham, MA, USA) at 30 °C.

2.10. Preparation of the selected bee pollen sample for rat experimentation

As explained, the current study was designed with twelve Anatolian bee-pollen samples at the initial stage defined as *in vitro* applications. The main aim of working with many bee-pollen samples in the preliminary trial as *in vitro* was to select the most correct product with the highest bioactivity for *in vivo* application. Thus, it was thought that the most accurate result would be obtained with a limited number of animals and the most ideal sample available. Briefly, according to the result of *in vitro* antioxidant analyses, the most effective bee pollen sample tagged as P5 Kocaeli was defined to use for animal application.

2.11. Statistical analysis

All results are presented as mean ± SEM. The data were analyzed with the SPSS (SPSS 24.0) program. The results are One-way ANOVA followed by Tukey's posthoc test to identify differences between groups. A value of $p < 0.05$ was considered statistically significant.

3. Result

3.1. Behavioral assessments

In the OFT in which anxiety-like behavior was evaluated, it was found that the time spent in the center of stress group rats significantly decreased (Table 1, $p < 0.05$). One of the anxiety-like behavior markers is that the rats spend more time in the margins than in the center. Pollen treatment, on the other hand, increased the time spent by stressed rats in the center ($p < 0.05$). Moreover, while locomotor activity, mean speed and grooming behavior decreased in stress group rats, immobilization time increased significantly. While pollen treatment increased locomotor activity, immobilization time and grooming behavior did not change significantly ($p > 0.05$). Our findings showed that chronic immobilization stress increased anxiety-like behaviors in rats, while bee pollen treatment decreased anxiety-like behaviors in rats. As shown in Table 2, in the FST, it was found that the immobilization time increased significantly only in the stress group compared to the control ($p < 0.05$). There was no significant difference between the swimming and climbing behavior parameters of the rats ($p > 0.05$). Our results showed that immobilization stress caused anxiety-like behavior rather than depression, while pollen treatment alleviates the negative effects of immobilization stress.

3.2. Biochemical measurements

Biochemical results are shown in Fig. 1. Accordingly, we observed that immobilization stress decreased BDNF level in hippocampus in rats (Fig. 1A, $p < 0.05$). However, bee pollen treatment markedly increased the BDNF level in hippocampal tissue. On the other hand, we analyzed proinflammatory cytokine markers in the study. We found that immobilization stress significantly increased TNF-α and IL-1β levels in the hippocampus compared to control ($p < 0.01$, $p < 0.05$, respectively). This result indicates an inflammation in the hippocampus. In contrast, we observed that bee pollen treatment caused a significant reduction in both IL-1β and TNF-α levels compared to the stress group (Fig. 1B-C, $p < 0.05$, respectively). On the other hand, we analyzed the levels of MDA and GSH, which are markers of oxidative stress in the hippocampus. Accordingly, we found that 2 h of immobilization stress increased MDA levels and decreased GSH levels in hippocampal homogenates (Fig. 1D-

Table 1
Scores of the Open Field Test (OFT).

	Control	Stress	Bee pollen + Stress
Total distance (m)	30.26 ± 1.01	22.30 ± 2.08^{*a}	27.42 ± 1.60^{**b}
Time spent in the central area (%)	6.22 ± 0.61	2.68 ± 1.16^{*a}	4.85 ± 0.95^{*b}
Total time immobility (s)	66.33 ± 5.22	106.43 ± 5.91^{*a}	95.68 ± 8.94
Number of entries to the central area	7.33 ± 1.09	5.83 ± 0.98	5.67 ± 0.80
Grooming frequency	2.2 ± 0.83	1.4 ± 0.14^{*a}	1.8 ± 1.30
Rearing frequency	3.6 ± 1.54	5.6 ± 2.40	5 ± 1.58

The data are present mean ± SEM. (n = 8). The differences between groups were evaluated using one-way ANOVA with *post hoc* Tukey test. *$p < 0.05$, **$p < 0.01$.
a, versus to control group.
b, versus to stress group.

Table 2
Scores of the Forced Swimming Test (FST).

	Control	Stress	Bee pollen + Stress
Immobility (s)	88.38 ± 11.17	139.52 ± 12.38*a	125.52 ± 13.11
Swimming (s)	137.22 ± 10.66	91.92 ± 6.85	83.78 ± 6.51
Climbing (s)	74.4 ± 3.73	68.56 ± 7.93	90.7 ± 3.95

The data are present mean ± SEM. (n = 8). The differences between groups were evaluated using one-way ANOVA with *post hoc* Tukey test. *p < 0.05.
a, versus to control group.

Table 3
The data of location and antioxidant properties of analyzed Anatolian bee pollen samples*

Code	Location	TPC (mg GAE/g) *	FRAP(μmol $FeSO_4{\cdot}7H_2O$/g) *
P1	Konya, Turkey	10.698 ± 0.105	41.307 ± 0.142
P2	Muğla, Turkey	9.214 ± 0.133	59.467 ± 0.059
P3	Kütayha, Turkey	9.643 ± 0.024	41.999 ± 0.404
P4	Kırşehir, Turkey	9.675 ± 0.147	57.078 ± 0.723
P5	**Kocaeli, Turkey**	**13.083 ± 0.025**	**74.860 ± 0.077**
P6	Manisa, Turkey	9.575 ± 0.097	63.456 ± 0.281
P7	Bursa, Turkey	12.362 ± 0.070	71.033 ± 0.047
P8	Aydın, Turkey	9.194 ± 0.063	69.268 ± 0.594
P9	Bayburt, Turkey	10.673 ± 0.028	35.638 ± 0.341
P10	Balıkesir, Turkey	10.644 ± 0.096	37.564 ± 0.293
P11	İstanbul, Turkey	10.989 ± 0.101	51.401 ± 0.678
P12	Alanya, Antalya, Turkey	9.015 ± 0.056	48.151 ± 0.057

*All results were performed in triplicate.

E, $p < 0.01$, respectively). However, we observed that bee pollen treatment decreased MDA and increased GSH compared to the stress group ($p < 0.05$, respectively).

3.3. *In vitro spectrophometric analyses of bee pollen*

The degree of *in-vitro* bioactivity properties of bee pollen samples was calculated by two assays as total phenolic content (TPC) and ferric reducing antioxidant power (FRAP), ranging from 9.015 to 13.083 mg GAE/g and from 35.638 to 74.860 μmol FeSO4·.7H2O/g, respectively (Table 3. Especially, the FRAP value is an important indicator to present the total antioxidant capacity of the studied sample. Besides FRAP, the value of TPC showed that the sample P5 from Kocaeli had the highest in the studied samples.

3.4. *The HPLC result of phenolic compounds of Kocaeli bee pollen sample*

It was decided to evaluate Kocaeli bee pollen sample in terms of phenolic compounds by HPLC because of its having the highest antioxidant value and being the main material for *in vivo* study. Moreover, it was believed that the current HPLC data could be a shred of evidence for the possible positive results of *in vivo* experiments. Hence, eighteen phenolic compounds were analyzed however nine of them were identified in varying amounts. The sample P5′s HPLC value expressed as μg phenolic compound/g sample was in the following descending order: benzoic acid, 26.493 ± 2.907 > quercetin, 10.254 ± 0.064 > syringaldehyde, 5.362 ± 0.226 > protocatechuic acid, 4.805 ± 0.337 > rutin, 2.445 ± 0.057 > catechin, 2.071 ± 0.282 > *p*-OH benzoic acid, 0.863 ± 0.068 > vanillin, 0.594 ± 0.088 > ferulic acid, 0.418 ± 0.032 (Table 4.

4. Discussion

This study is the first to show the beneficial effects of bee pollen on both neurochemical and anxiety-like behavior in neuronal insufficiency and behavioral defects caused by chronic immobilization stress in adult rats. A 10-day stress exposure was found to induce neuroinflammation and oxidative stress while decreasing the hippocampal neurotrophin level in rats. We found that bee pollen increased BDNF level in the hippocampus of rats treated with immobilization stress while reducing inflammation and oxidative stress. Our results provide evidence to support the neuromodulatory potential of bee pollen. The current evidence shows that bee products are an influential antioxidant source. Especially, bee pollen is widely preferred is known as a food supplement thanks to having potential bioactive substances that are responsible for biological properties such as antioxidant effect [25]. The current determination could be seen as a correlation study because of showing potential accordance with data of the literature to support this claim. In this study, TPC results of twelve samples supplied from different locations of Anatolia, Turkey are in almost concordance with those reported by recent studies [26–27]. When evaluated to the FRAP results found in the literature, it was noticed that the current results nearly harmonized with previous ones just like TPC data [26–28].

Phenolic compounds are generally classified into two sub-groups as phenolic acids and flavonoids; these directly influence the activity level of bio-properties [24]. Bee pollens include various types and amounts of phenolic compounds that depend on the source from which this has been supplied [25]. It can be easily observed the differentiation of types and amounts in previous studies related to the phenolic evaluation of bee pollens. Hemmami et al. [29] analyzed nine phenolic compounds identified as gallic acid, chlorogenic acid, vanillic acid, caffeic acid, vanillin, *p*-coumaric acid, rutin, naringin, and quercetin in 13 methanolic pollen extracts from different regions of Algeria. According to this study, gallic acid, *p*-coumaric acid, and quercetin were detected as different amounts in all analyzed pollen samples. The phenolic content of bee pollen used in the study was determined in different types and amounts similar to other studies. Consequently, it was well known the main reason for the differentiation of the chemical composition -also including phenolics- of bee pollen samples was some parameters such as the botanical variety, storage time, and environmental conditions, etc. [25,30].

Increasing evidence has shown that chronic immobilization/restraint stress can trigger anxiety and depression-like behavior in rodents [16,31]. In our study, we observed that rats exposed to stress for 2 h a day for 10 consecutive days were more anxious than the control. On the other hand, we found that bee pollen treatment reduced anxiety-like behavior but did not significantly affect depression-like behavior. A previous study reported the anxiolytic effect of bee pollen in mice [13]. Our experimental results revealed the potential anxiolytic effect of bee pollen in immobilization stress in rats. Many studies have shown that restraint stress increases cognitive dysfunction and anxiety-like behaviors [2,16,32]. This study shows that bee pollen prevents cognitive impairment and anxiety-like behaviors due to immobilization stress in rats by modulating BDNF, TNF-α and IL-1β. BDNF, a neurotrophic factor, plays an important role in the maintenance, survival, differentiation and growth of neurons increase is important for learning and memory development [33]. Our findings show that immobilization stress decreases hippocampal BDNF level, but bee pollen administration has a significant increase in BDNF level. Liao et al. [12] reported that the BNDF level in the hippocampus increased in mice treated with bee pollen against scopolamine-induced cognitive impairment. Taken together, we express that bee pollen can exert beneficial effects by regulating the hippocampal BDNF level.

On the other hand, there is evidence that increased brain cytokines due to repetitive stress stimuli play an important role in the etiology of depression and anxiety.

Recent studies have shown that restrictive stress-induced anomalies may be associated with hippocampal inflammation and oxidative stress [34–36]. Our findings showed that exposure to repeated stress increased hippocampal TNF-α and IL-1β levels in rats. This result indicates the role of neuroinflammation in the etiology of anxiety-like behavior. It also supports the results of some previous studies [36–37]. In this study, we found that bee pollen treatment significantly reduced TNF-α and IL-1β

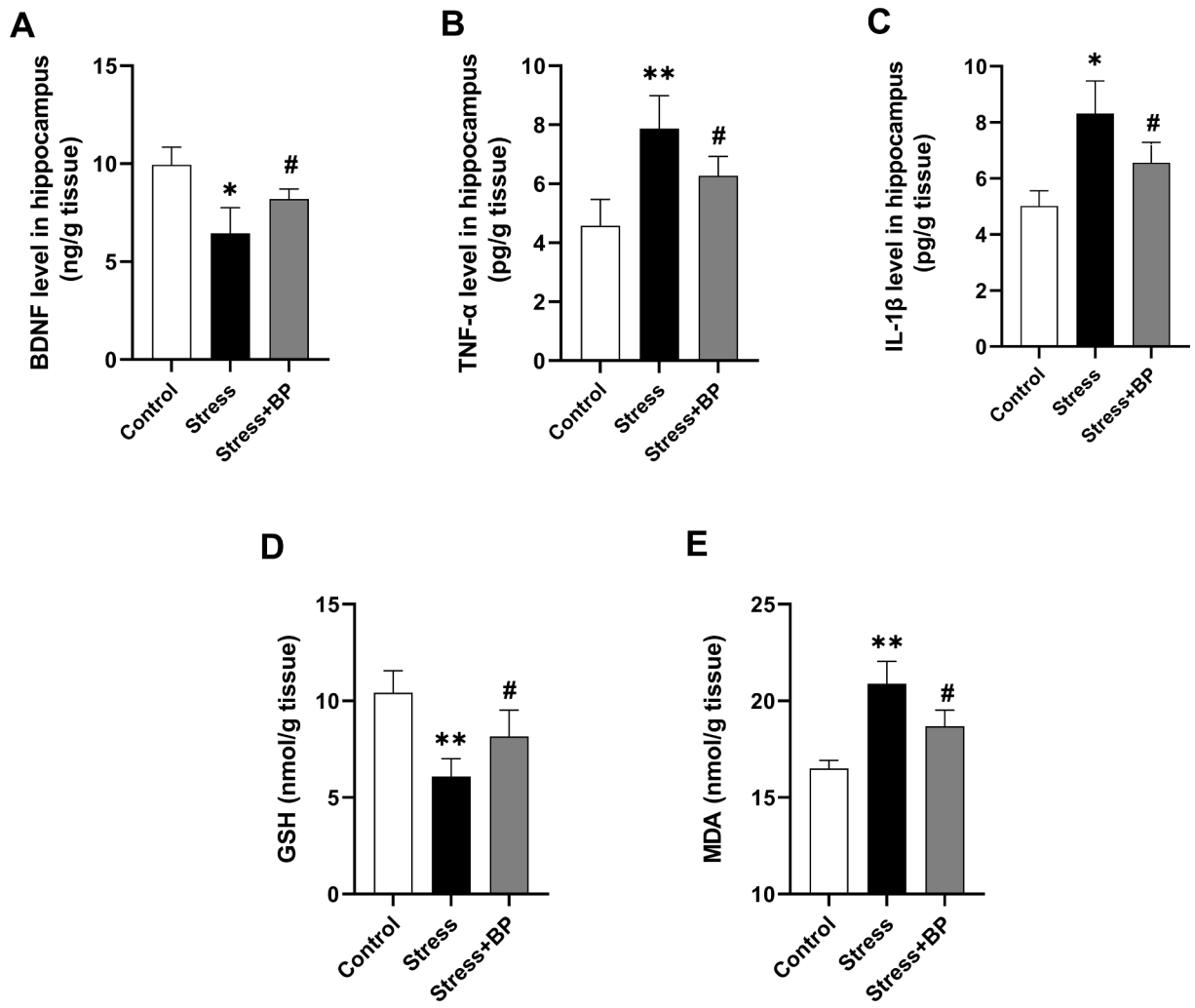


Fig. 1. The effect of bee pollen on hippocampal A) BDNF, B) TNF-α, C) IL-1β, D) GSH and E) MDA levels in rats exposed to chronic immobilization stress. *p < 0.05 vs. control, ≠p < 0.05 vs. stress and **p < 0.01 vs. control. The data are present mean ± SEM (n = 8).

Table 4
The phenolic profile of the selected bee pollen sample (P5, Kocaeli).

No	Phenolic Compounds	μg phenolic compound/g sample*
1	Gallic Acid	N.D.
2	Protocatechuic Acid	4.805 ± 0.337
3	Protocatechuic Aldehyde	N.D.
4	*p*-OH Benzoic Acid	0.863 ± 0.068
5	Catechin	2.071 ± 0.282
6	Chlorogenic Acid	N.D.
7	Vanillic Acid	N.D.
8	Caffeic Acid	N.D.
9	Syringic Acid	N.D.
10	Epicatechin	N.D.
11	Vanillin	0.594 ± 0.088
12	*p*-coumaric Acid	N.D.
13	Syringaldehyde	5.362 ± 0.226
14	Rutin	2.445 ± 0.057
15	Ferulic Acid	0.418 ± 0.032
16	Benzoic Acid	26.493 ± 2.907
17	Rosmarinic Acid	N.D.
18	Quercetin	10.254 ± 0.064

*In all the results given, the analyses were performed in triplicate; N.D.: Not detected.

levels in the hippocampal tissues of stressed rats. Several previous studies have reported the regulation and anxiolytic effect of bee pollen on neurocognitive functions [12–13]. However, in our study, the role of bee pollen treatment in reducing the anomalies caused by repetitive stress has been demonstrated for the first time. Therefore, our results could demonstrate the potential role of bee pollen in the prevention of stress-induced neuroinflammation. Immobilization stress has been known to induce oxidative damage in several tissues such as the brain, kidney, and liver. The brain cell membrane is very sensitive to lipid peroxidation caused by oxidative stress since it contains large amounts of polyunsaturated lipids. The level of MDA, which is a marker of lipid peroxidation, increases with increasing lipid degradation in the brain [38]. As a matter of fact, our study showed that the MDA level in the hippocampus increased as a result of 2 h immobilization stress.

4.1. Limitations

Here we report that our study has some limitations. The primary focus of our study was anxiety/depression-like behavior, neurotrophic factor, and hippocampal neuroinflammation. Therefore, we could not examine the efficacy of stress-related genes, cholinergic system and neurotransmitters in the same experimental setup. Moreover, although the duration of immobilization stress and the drug doses used were selected from the current literature [2,14], we suggest investigating the effectiveness of different stress durations and drug doses in new studies.

5. Conclusion

This study is the first to demonstrate the anxiolytic effect of bee pollen against behavioral disorders caused by immobilization stress in rats. We observed that repetitive immobilization stress decreased the level of neurotrophic factor in the rat hippocampus and increased inflammation and oxidative stress, while bee pollen treatment showed an anxiolytic effect by significantly reversing these deteriorating effects. In this context, we suggest that bee pollen may be a potential therapeutic intervention in the prevention or alleviation of stress-related behavioral deficiencies.

CRediT authorship contribution statement

Özlem Saral: Conceptualization, Methodology, Investigation, Writing – original draft, Project administration, Writing – review & editing. **Hüseyin Şahin:** Investigation, Resources, Writing – original draft, Writing – review & editing. **Sinan Saral:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Project administration. **Mehmet Alkanat:** Formal analysis, Software, Data curation. **Kerimali Akyıldız:** Resources, Formal analysis, Investigation. **Atilla Topçu:** Resources, Investigation. **Adnan Yılmaz:** Resources.

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