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Research Paper / Araştırma Makalesi

Effect of Black Mulberry (*Morus nigra*) Consumption on Antioxidant Capacity and Inflammation in Patients Mild-to-Moderate Alzheimer's Dementia: A Randomized Clinical Pilot Study

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ABSTRACT

This study aimed to determine the effect of black mulberry (*Morus nigra*) on antioxidant capacity and inflammation in patients with Alzheimer's Dementia (AD). We randomly assigned older adults with mild-to-moderate AD (n=44) to the intervention or control group. An intervention group was administered 20 g of black mulberry concentrate daily for 12 weeks. This study was completed by 20 participants in the intervention group and 19 in the control group. At the beginning and end of the study, the serum levels of superoxide dismutase (SOD) enzyme, Total Oxidant Status (TOS), Total Antioxidant Status (TAS), and oxidative stress index were determined. To evaluate inflammation, serum Interleukin 1-beta (IL-1 beta) and Transforming Growth Factor-beta (TGF-β) levels were measured. Furthermore, 3-day food records were obtained and dTAC was calculated on the basis of oxygen radical absorption capacity (ORAC), Trolox® equivalent antioxidant capacity (TEAC), Total Radical Trapping Antioxidant Parameter (TRAP), Ferric Reducing Antioxidant Potential (FRAP) and Total Phenolic (TP). There were no significant changes in SOD, TGF-β, IL-1 and TAS serum levels from baseline to the end of the study. However, a statistically significant increase was found in the TOS and oxidative stress index levels for only control group ($p<0.05$). There were significant increases in the intervention group for dietary T-ORAC, H-ORAC, TEAC, TRAP, FRAP-2, FRAP-3, and dTAC ($p=0.003$). However, no significant relationship was found between serum inflammation markers and dietary antioxidants or dTAC. In conclusion, black mulberry had a limited effect on antioxidant capacity and inflammation in older adults with mild-to-moderate AD.

Key words: Anthocyanin, Alzheimer disease, Black mulberry, Flavonoid, Antioxidant, Inflammation

Hafif-Orta Derecede Alzheimer Demanslı Hastalarda Karadut *(Morus nigra*) Tüketiminin Antioksidan Kapasite ve İnflamasyon Üzerine Etkisi: Randomize Klinik Pilot Çalışma

ÖZ

Bu çalışmanın amacı Azheimer demansı olan hastalarda; karadut (*Morus nigra*) konsantresinin antioksidan kapasiteye ve inflamasyona etkisini incelemektir. Araştırma hafif-orta evre Alzheimer demansı olan 44 birey üzerinde yürütülmüştür. Müdahale grubuna 12 hafta boyunca günde 20 g karadut konsantresi verilmiştir. Çalışma 20 kişilik müdahale grubu, 19 kişilik kontrol grubu ile tamamlanmıştır. Araştırmanın başında ve sonunda antioksidan kapasitenin değerlendirilmesi için Süperoksit dismutaz (SOD) enzimi, Total Oksidan seviyesi (TOS), Total Antioksidan Seviyesi (TAS) ve oksidatif stres indeksi düzeylerine bakılmıştır. İnflamasyonu değerlendirmek için serumda İnterlökin 1-Beta (IL-1 beta) ve Transforme Edici Büyüme Faktör Beta (TGF-beta) seviyelerine bakılmıştır. Ayrıca hastalardan 3 günlük besin tüketim kayıtları alınarak; Oksijen radikali absorbans kapasitesi (ORAC), Trolox® eşdeğeri antioksidan kapasite (TEAC), Total radikal

yakalama antioksidan kapasitesi (TRAP), Demir iyonu indirgeyici antioksidan güç (FRAP), Toplam Fenolik (TP) değerleri temel alınarak diyetin toplam antioksidan kapasitesi (dTAC) hesaplanmıştır. SOD, TGF-beta, TAS, IL-1 beta düzeylerinde anlamlı bir değişiklik olmamıştır. Ancak sadece kontrol grubunda TOS ve oksidatif stres indeksi düzeylerinde istatistiksel olarak anlamlı artış tespit edilmiştir ($p<0,05$). Diyet T-ORAC, H-ORAC, TEAC, TRAP, FRAP-2, FRAP-3 ve dTAC düzeylerinde müdahale grubunda anlamlı artış görülmüştür ($p<0,05$). Ancak, serum inflamasyon belirteçleri ile diyet antioksidanları veya toplam diyet antioksidan kapasitesi (dTAC) arasında anlamlı bir ilişki bulunmamıştır ($p>0,05$). Sonuç olarak karadutun hafif-orta şiddette Alzheimer demansı olan yaşlılarda antioksidan kapasite ve inflamasyon üzerinde sınırlı etkiye sahip olduğu bulunmuştur.

Anahtar Kelimeler: Antosiyanin, Alzheimer hastalığı, Karadut, Flavonoid, Antioksidan, İnflamasyon

INTRODUCTION

Alzheimer's disease is a progressive neurodegenerative disorder characterized by cognitive decline, memory impairment, and behavioral changes [1, 2]. It is the most common cause of dementia worldwide, affecting an estimated 55 million people, with prevalence increasing with age [1]. Amyloid β (Aβ) is at the center of the inflammatory process in Alzheimer's disease. Aβ; activates microglia and astrocytes that increase the secretion of pro-inflammatory molecules such as cytokines, chemokines, prostaglandins, and free radicals in the central nervous system. Although microglial activation increases Aβ clearance, decreases glutamate-related neurotoxicity, and has a positive effect on neurogenesis, these effects are prevented by excessive secretion of microglial cells [3]. Increased inflammation is also associated with increased reactive oxygen species (ROS) production. Overproduction of ROS is associated with activation of microglial cells as well as activation of inflammatory mediators such as inducible nitric oxide synthase (iNOS), interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and nuclear factor kappa B (NF-κB) in many inflammatory diseases [4].

The brain is particularly sensitive to oxidative damage due to its high rate of oxygen consumption. In addition, the activity of endogenous antioxidant enzymes (such as superoxide dismutase, catalase, glutathione peroxidase, and heme-oxygenase) seems to be decreased in Alzheimer's patients [5] Furthermore, as the brain ages, it becomes more vulnerable to oxidative stress [4]. Triggering oxidative stress in the brain causes mitochondrial dysfunction and the accumulation of Aβ [5]. In Alzheimer's disease, there is an increase in iron and copper, which are redox metals that can form ROS. In this process, there is a decrease in cytochrome C oxidase, an increase in advanced glycation end products (AGE), protein carbonyl (PCO) compounds, 8-hydroxyguanine (8-OHG), malondialdehyde (MDA), peroxynitrite levels, and the production of pro-inflammatory cytokines such as IL-6 [4, 5].

Polyphenols, which are one of the major groups of phytochemicals, are components that are vital to human health [6]. Anthocyanins, flavanols, flavanones, flavones, flavanols, and isoflavones are the six subgroups that make up the subclass of polyphenols known as flavonoids. A subclass of flavonoids derived from polyphenolic substances called anthocyanins is widely distributed in a variety of flowers, leaves, seeds, and fruits, including berries [7].

Anthocyanins have a high oxygen radical absorption capacity (ORAC) value [6]. Anthocyanins; can directly modulate the damage caused by reactive oxygen and nitrogen species (ROS and RNS) by increasing glutathione peroxidase (GPx) activity and nuclear factor erythroid 2–related factor 2 (Nrf_2) activity, as well as by improving mitochondrial functions [9]. *In vitro* studies showed that anthocyanin supplementation can reduce the release of pro-inflammatory proteins such as nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are increased in response to LPS-induced stimulation [10, 11]. Furthermore, treatment with anthocyanin-rich extracts was found to reduce the production and release of nitric oxide, IL1β, and TNF-α [10, 11]. While neuro-inflammation studies mainly focus on the inflammation of microglia in the central nervous system, reactive astrocytes can secrete many of the same inflammatory factors as microglia, and studies have found that the LPS-induced inflammatory response in astrocytes is significantly reduced as a result of treatment with anthocyanin-rich extracts [12, 13].

Black mulberry is rich in polyphenols, flavonoids, and anthocyanins, which are responsible for their antioxidant and anti-inflammatory activities [14].Therefore, we hypothesized that black mulberry consumption could help to improve inflammatory conditions and increase antioxidant capacity in the early stages of dementia. The purpose of this study was to investigate the effects of black mulberry concentrate on antioxidant capacity and inflammation in patients with Alzheimer's disease.

MATERIALS and METHODS

Trial Design

This study was designed as a two-group randomized clinical pilot study and registered on clinicaltrials.gov with registration number NCT05406648. This study was approved by the ethics committee of Ankara Numune Education and Research Hospital (date: 02/07/2018 and number:18-2325) and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Further permits and clinical trial registration were acquired from the Ministry of Health (Number: 66175679-514.11.01-E.33211 Clinical Research 8-AKD-167, date: 10/04/2019), (Number: 50687469-799, date: 06/20/2019). Written informed consent was obtained from all subjects.

Participants

Sample size was determined via power analysis using anticipated means in accordance to the previous research data with 90% confidence interval and 0.05 type-I error (G*Power software, version 3.1.9.2, Franz Faul, Universitat Kiel, Dusseldorf, Germany). A total of 44 subjects (aged over 65 years) with mild-to-moderate Alzheimer's disease, who applied to a tertiary geriatric outpatient clinic were included in this study. The participants were split into two groups at random: intervention (n=22) and control (n=22). One participant in the intervention group left the study owing to unrelated health problems, and another participant left the study because they were unable to ingest mulberry concentrate. In addition, two people in the control group discontinued the study for personal reasons, and one subject left the study due to unrelated health difficulties. The study was completed with 20 participants in the intervention group and 19 in the control group (Figure 1). The gathering of the data commenced on August 8, 2019, and ended on March 17, 2020.

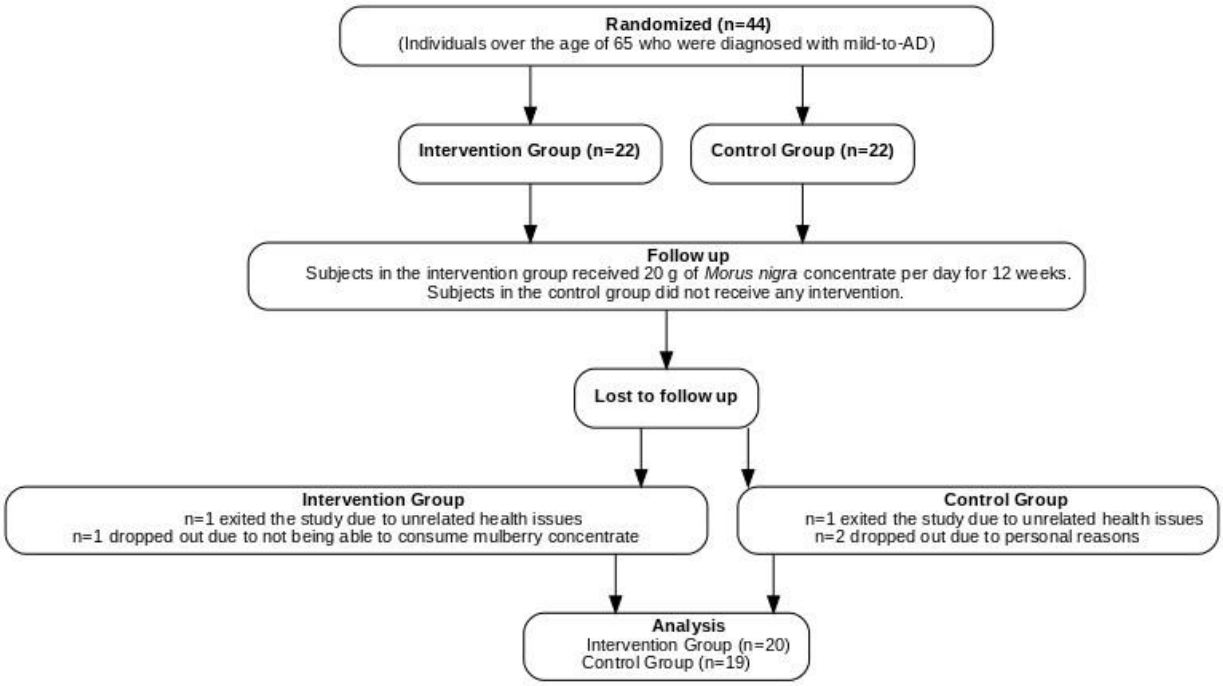


Figure 1. Study flow diagram

After the working conditions were specified, the 'Informed Consent Form' was read and signed by the individuals who volunteered to participate. Volunteers within the study are in a position to give written consent if there is no additional justification. However, for patients who were not able to answer the questions, the 'Informed Consent Form' was read and signed by their guardians.

Following were the criteria for inclusion: AD of mild to moderate severity diagnosed within the first phase; anti-dementia or anti-depressant treatment, if any, started at least 6 months prior to the study and continued at the same dosage for at least 3 months; no significant change in the patient's regular medical care during the study; no use of anthocyanin supplements in the 4 months prior to the study baseline; and at least one caregiver who could follow daily concentrated *Morus nigra* consumption. The following conditions were required for exclusion: being under 65 years old; residing in a nursing home; suffering from cancer, uncontrolled hypertension or diabetes, an autoimmune disease that has not been identified, major depression, alcohol or drug addiction; a history of delirium, a feeding tube, becoming bedridden; and taking medications that could influence the outcome of the study, such as benzodiazepines and antipsychotics. Additionally, patients who might be at risk for p-glycoprotein/CYP3A/*Morus nigra* nutrient-drug interactions due to their medication use were not included in the study groups. These pharmaceuticals include calcium channel blockers (such as amlodipine), calcineurin inhibitors (such as tacrolimus and cyclosporine), antineoplastic agents (docetaxel, etoposide, and vincristine), digoxin, macrolide antibiotics (such as clarithromycin), and protease inhibitors.

Randomization and Study Protocol

Block randomization was utilized in this study to create the groups in order to avoid selection bias. A random number generator was used to generate the subject numbers, which ranged from 1 to 44. The initial 22 strings of numbers were given to the intervention group, whereas the next 22 strings of numbers were given to the control group.

For 12 weeks, the participants in the intervention group consumed 20 g of *Morus nigra* concentrate each day. The subjects in the control group received no treatment. Both

groups of patients continued their regular medical care as prescribed by their doctors. The anthocyanin content of the black mulberry concentrate used in this study was determined to be 1090.814 ± 0.4905 mg/kg. Based on analyses of both raw material and final product, a daily dose of 20 g concentrate (equivalent to approximately 165 g fresh fruit) was selected, providing 22 mg anthocyanins. According to a review [43], analysis of NHANES 2001–2002 data indicated that the average daily anthocyanin intake in the U.S. population was 12.5 mg, with seasonal totals of 215 mg in summer and 180 mg in winter. The anthocyanin intake in our study exceeds these averages. Although no clinical trial has investigated black mulberry in dementia patients, a randomized controlled trial in hypercholesterolemic subjects reported that consuming 45 g/day freeze-dried mulberries (≈160 g fresh fruit) for six weeks significantly reduced total cholesterol and LDL, while increasing antioxidant markers [44]. In the absence of established dosage and duration recommendations for mulberries due to limited human studies [45], we adopted a 20 g/day dose for 12 weeks based on anthocyanin content, equivalence to fresh fruit, and prior evidence of efficacy in related populations.

At the baseline and end of the study, participants were requested to attend the clinic twice. The participants and their caregivers in the intervention group were given a thorough explanation of the intervention approach at baseline following the clinical examination. At the baseline visit, with the help of their caregivers, all participants were given a questionnaire that contained general demographic data. All patients in both groups had anthropometric measurements and blood sampling on both visits. At the beginning and at the end of the study, in order to evaluate antioxidant capacity in serum samples SOD enzyme, TOS, TAS, and oxidative stress index level were examined. For the evaluation of inflammation, serum Interleukin 1-beta (IL-1 beta) and transforming growth factor beta (TGF-beta) levels were measured. In addition, anthropometric measurements were taken from the patients at the beginning, and end of the study. Also, at the beginning and end of the study 3-day individual food consumption record were obtained and dTAC was calculated on the basis of ORAC, TEAC, TRAP, TP, and FRAP. FRAP values were assessed from multiple sources and named as FRAP-1 [15], FRAP-2 [16], FRAP-3 [17, 18], and FRAP-4 [19].

Anthropometrics Measurements

Body Weight and Body Mass Index

Participants were requested to take off their heavy outerwear, including their coat, jacket, and shoes. A digital scale accurate to 0.1 kg was used to determine the body weight. Each participant's overall weight was reduced by 0.5 kg to account for the remaining clothing. Body mass index (BMI) was calculated using the formula: body weight (kg)/height (m).

Hand Grip Strength

A hand dynamometer was used to gauge the strength of the participants' hands grip. (Takei 5401, Takei Scientific Instruments Co., Ltd, Tokyo, Japan). The subjects stood while being measured with their wrists and elbows completely extended. The measurements were performed three times with 15-second gaps, and the mean values were computed and recorded.

Dietary Intake

At the beginning and the end of the study, 3-day individual food consumption records were collected to determine the dietary intake of the participants. Total antioxidant capacity of the diet was determined by calculating FRAP, TEAC, hydrophilic ORAC (HORAC), total ORAC (TORAC) and TRAP of food items. For the FRAP method, the antioxidant content of various foods was evaluated by FRAP analysis in the database created by Carlsen et al. (FRAP 1) [15] and Pellegrini et al. (FRAP 2) [18]. Furthermore, for TEAC and TRAP calculations the database created by Pellegrini et al.[18] were used. The ORAC method has three sub-methods: hydrophilic (HORAC) and total (TORAC). In the database created by the United States Department of Agriculture (USDA), the antioxidant contents of various foods (326 foods) were evaluated by HORAC and TORAC analyses [15, 17-19, 21-23]. While determining the total antioxidant capacity of the diets; FRAP1, FRAP 2, TRAP, TEAC and HORAC and TORAC values for each food in the aforementioned databases were calculated based on the food intake records.

Diagnosis of Alzheimer's Disease

Blood testing, physical and mental status assessments, history collection, physical examinations, and, if necessary, radiographic investigations were all part of the clinical evaluation of dementia (computed tomography or magnetic resonance imaging). Using the criteria of the National Institute on Aging and the Alzheimer's Association (NIA-AA) and the Clinical Dementia Rating scale, an expert geriatrician determined the diagnosis and severity of AD [24, 25].

Serum Proteins

Blood samples were collected for serum SOD, IL-1beta, TGF beta, MDA protein analysis during all two visits. Samples were centrifuged at 3000 rpm for 10 minutes (Heraeus Labofuge 200 Centrifuge, USA) and stored at −80°C until subsequent analysis. IL-1 beta (Catalog No: KAP1211, Belgium), SOD (Catalog No: E0700Hu, China), MDA (Catalog No: E1371Hu, China), TGF beta (Catalog No: BMS249-4 and BMS249-4TEN, Austria) proteins were measured by using commercially available human Enzyme Linked Immunosorbent Assay (ELISA) kits following manufacturer's protocol at Hacettepe University Department of Nutrition and Dietetics Scientific Research Laboratory. All samples were tested as duplicates. Serum samples for TGF beta were diluted 1:10. No dilution was made for other proteins.

Total Antioxidant Level, Total Oxidant Level and Oxidative Stress

Total antioxidant and total oxidant levels were determined calorimetrically using Rel Assay Diagnostics (Ankara, Türkiye) kits. The measurement of total antioxidant capacity is based on the reduction of the colored ABTS (2,2'-azino-bis 3 ethylbenzothiazole-6-sulfonic acid) cationic radical by all antioxidant molecules in the samples and the subsequent decolorization of the colored radical in proportion to the sum of the antioxidant molecules in the sample. Oxidative Stress Indices (OSI) were obtained as a percentage of the ratio of total oxidant status to total antioxidant status. Before calculation, the unit of the Total Antioxidant Capacity (TAC) test was converted to the unit of the TOS test [26].

$$OSI = \left(\frac{TOS\ \mu mol\ H_2O_2 \frac{Equivalent}{L}}{TAC\ mmol\ Trolox \frac{Equivalent}{L}} \right) * 100$$

Intervention

Morus nigra

In the Döhler factory in Karaman (Türkiye), black mulberries gathered in Ayaş (Ankara, Türkiye) area were concentrated by being squeezed, pressed, and put through a coarse filter. The anthocyanin content of the black mulberry concentrate utilized in the study was estimated to be 1090.814±0.4905 mg/kg based on the analysis results. Each person received 90 unique containers in total throughout the course of three visits (30 containers per visit). Caretakers were instructed to store the concentrate containers at -18 to -20°C and move each container to the +4°C section 1 day before consumption in order to reduce nutritional loss.

Outcomes

The primary outcomes of the study were determined as individuals leaving the study voluntarily, not complying with the study protocol, and not being able to continue the application of black mulberry concentrate. The secondary outcomes of the study were determined as reaching the number of samples in which the effects of black mulberry concentrate use on cognitive functions could be evaluated. In addition, the research protocol was planned as to be terminated if improvement was observed in the experimental group before the 3 months of planned treatment.

Statistical Analysis

SPSS was used to perform the statistical analysis (IBM SPSS 25.0, Armonk, NY: IBM Corp.). Using predicted means with a 90% confidence interval and a 0.05 type-I error, the sample size was calculated using power analysis (G*Power software, version 3.1.9.2, Franz Faul, Universitat Kiel, Dusseldorf, Germany). The Kolmogorov-Smirnov test was used to determine whether the distribution was normal. Parametric variables with a normal distribution were subjected to an independent sample t-test and a paired sample t-test. The Pearson correlation test was used to examine correlation estimation. Using the chi-square test, the relationship between qualitative variables was investigated. The statistical significance threshold was set at a P value of 0.05, and the results were reported as means and standard error of measurement (SEM).

RESULTS

In this study, 59% of the individuals were male (mean age 81.4±1.22 years) and 41% were female (mean age: 79.2±1.63 years). Table 1 shows the changes in the anthropometric measurements after 12 weeks of intervention.

Table 1. Changes in anthropometric measurements after 12 weeks of intervention*

	Women			Men		
	Intervention group (n=7)	Control group (n=9)	P_a	Intervention group (n=20)	Control group (n=19)	P_a
Body weight (kg)						
Baseline	63.5±4.65	67.8±3.43	>0.05	71.5±2.03	77.6±3.02	>0.05
Change 0-12 weeks	0.8±0.86	0.7±0.94		1.1±0.75	1.4±1.31	
BMI (kg/m^2)						
Baseline	27.5±1.56	28.0±1.21	>0.05	26.1±0.83	26.7±0.96	>0.05
Change 0-12 weeks	0,4±0.34	0.2±0.39		0.4±0.27	0.3±0.58	
Hand Grip Strength						
Baseline	14.0±4.09	17.0±2.89	>0.05	20.2±5.80	22.7±5.19	>0.05
Change 0-12 weeks	1.0±3.48	-1.1±2.43		0.3±4.42	-0.25±4.15	
Waist Circumference (cm)						
Baseline	100.3±4.48	99.5±3.32	>0.05	94.9±6.4	102.5±2.27	>0.05
Change 0-12 weeks	-4.3±2.57	0.3±1.35		0.5±1.29	1.2±1.42	
Mid-upper Arm Circumference (cm)						
Baseline	27.6±1.75	29.1±1.28	>0.05	31.6±4.18	28.8±0.85	>0.05
Change 0-12 weeks	-0,3±0.74	-0.2±0.74		0.8±0.68	-0.9±0.82	

*: Data are given as mean ± SD. Baseline refers to measures obtained at the pre-intervention assessment. P_a: denotes differences between the 2 groups using an independent t-test. BMI: Body Mass Index.

There was no statistical difference between the control and intervention groups for any of the anthropometric measurements (p>0.05). Dementia was diagnosed on average 35.3±8.89 months ago in the intervention group and 26.1±4.63 months ago in the control group. Table 2 shows the serum protein levels at baseline and after 12

weeks of intervention. In serum SOD, TGF- β, and TAS levels, there was no statistically significant change. For TOS and oxidative stress index levels, a significant increase was found for only the control group ($p<0.05$).

Table 2. Serum protein levels at baseline and after 12 weeks of intervention*

	Intervention Group (n=20)	P_a	Control Group (n=19)	P_a	P_b
SOD (ng/mL)					
Baseline	21.3±3.23	0.400	18.1±2.76	0.447	
After 12 weeks	19.8±1.98		19.3±3.98		
Δ 0-12 weeks	-1.4±1.65		1.13±1.45		0.256
IL-1β (pg/mL)					
Baseline	7.4±0.54	<0.001	9.2±1.05	0.014	
After 12 weeks	4.2±0.55		6.1±1.15		
Δ 0-12 weeks	-3.1±0.64		-3.0±1.11		0.924
TGF-β (pg/mL)					
Baseline	1253.2±70.38	0.884	1760.8±91.88	0.701	
After 12 weeks	1270.2±83.94		1929.4±429.5		
Δ 0-12 weeks	17±115.37		168.6±432.12		0.731
TOS (μmol/L)					
Baseline	6.6±0.59	0.698	5.5±0.52	0.005	
After 12 weeks	6.3±0.42		8.2±0.67		
Δ 0-12 weeks	-0.30±0.78		2.6±0.81		0.013
TAS (μmol/L)					
Baseline	1.3±0.23	0.105	1.3±0.28	0.104	
After 12 weeks	1.2±0.25		1.2±0.24		
Δ 0-12 weeks	-0.10±0.24		-0.08±0.19		0.737
Oxidative Stress index					
Baseline	0.04±0.004	0.356	0.04±0.005	0.004	
After 12 weeks	0.05±0.004		0.07±0.005		
Δ 0-12 weeks	0.006±0.006		0.02±0.007		0.072

*: Data are given as mean ± SD. Baseline refers to measures obtained at the pre-intervention assessment. P_a: Paired t test was used for analysis to compare the same group before and after intervention. P_b: Denotes differences between the 2 groups using an independent t-test.

Table 3 shows the contribution of food groups to dietary total antioxidant capacity in intervention and control groups at baseline and after 12 weeks of intervention. There were significant increases in the intervention group for dietary T-ORAC, H-ORAC, TEAC, TRAP, FRAP-2, FRAP-3, TP, and dTAC between baseline and the 12th week ($p=0.003$). However, these values in the control group did not show any significant difference. Table 4 shows the correlation of differences in dietary antioxidants, dTAC, and serum inflammatory markers from baseline to the 12th week in the entire study population. There was a significant negative correlation between the difference in TOS scores and the differences in FRAP-2, FRAP-3, and TP values ($p=0.039$, $p= 0.047$, and $p=0.036$; respectively).

DISCUSSION

In this study, which, as far as we know, was the first study to administer *Morus nigra* to AD patients for 12 weeks, we observed significant increases in dietary T-ORAC, H-ORAC, TEAC, TRAP, FRAP-2, FRAP-3, TP, and dTAC levels in intervention group; significant increases in TOS and oxidative stress index levels in the control group although no significant changes in anthropometric measurements and serum SOD, TGF- β, and TAS levels in either of the groups, from baseline to 12th week of intervention. Furthermore, when all patients analyzed together there was a negative correlation between the difference in TOS scores and the differences in FRAP-2, FRAP-3, and TP values.

In the present study, we observed that after 12 weeks of black mulberry consumption, TOS levels significantly increased in the control group while showing a decrease in the intervention group. Although the decrease in TOS in the intervention group was not significant, it may indicate that black mulberry concentrate may have a protective effect against TOS. Additionally, our results suggest that black mulberry consumption may increase dietary T-ORAC, H-ORAC, TEAC, TRAP, FRAP-2, FRAP-3, TP, and dTAC. However, no significant relationship was found between serum inflammation markers and dietary antioxidants or dTAC. In the EPIC study, conducted to determine the effect of dietary antioxidants on serum inflammatory indicators, no significant relationship was found between serum inflammation markers and dietary flavonoids, including β-carotene, retinol, α-tocopherol, vitamin C, TEAC, TRAP, and FRAP [27].

Table 3. Dietary total antioxidant capacity in intervention and control groups at baseline and after 12 weeks*

	Intervention group (n=20)	P_a	Control group (n=19)	P_a	P_b
T-ORAC (µmol TE)					
Baseline	16633.3±1456.74	<0.001	16361.4±1539.72	0.798	
After 12 weeks	42450.7±1950.74		16693.1±1191.86		
Change 0-12 weeks	25817.5±1667.09		331.7±1270±42		<0.001
H-ORAC (µmol TE)					
Baseline	14911.9±1316.48	<0.001	14598.8±1228±29	0.564	
After 12 weeks	40514.5±1772±54		15170.8±992.83		
Change 0-12 weeks	25602.6±1439.64		572.1±967.51		<0.001
TEAC (mmol TE)					
Baseline	2.6±0.24	<0.001	3.5±0.46	0.552	
After 12 weeks	6.6±0.44		3.3±0.45		
Change 0-12 weeks	4.1±0.37		-0.2±0.29		<0.001
TRAP (mmol TE)					
Baseline	2.8±0.33	<0.001	3.7±0.76	0.285	
After 12 weeks	6.7±0.56		3.4±0.58		
Change 0-12 weeks	4.0±0.39		-0.4±0.32		<0.001
FRAP-1 (mmol)					
Baseline	4.1±0.52	0.840	3.6±0.34	0.795	
After 12 weeks	4.2±0.44		3.6±0.34		
Change 0-12 weeks	0.2±0.70		0.1±0.19		0.901
FRAP-2 (mmol)					
Baseline	2.6±0.19	<0.001	2.8±0.23	0.227	
After 12 weeks	13.0±0.35		2.6±0.23		
Change 0-12 weeks	10.5±0.37		-0.2±0.16		<0.001
FRAP-3 (mmol)					
Baseline	6.7±0.82	<0.001	7.8±0.94	0.958	
After 12 weeks	16.9±1.28		7.7±1.16		
Change 0-12 weeks	10.2±0.98		-0.0±0.83		<0.001
FRAP-4 (mmol)					
Baseline	3.3±0.37	0.056	5.2±0.63	0.175	
After 12 weeks	5.3±0.87		4.6±0.55		
Change 0-12 weeks	1.9±0.92		-0.6±0.43		0.019
DTAC (mg/100g)					
Baseline	189.7±31.67	0.004	200.1±45.86	0.190	
After 12 weeks	281.5±28.54		227.8±47.50		
Change 0-12 weeks	91.8±26.38		27.7±20.14		0.064
TP (µmol TE/100g)					
Baseline	1177.7±183.75	<0.001	1049.1±115.90	0.273	
After 12 weeks	4445.5±184.98		1129.2±110.60		
Change 0-12 weeks	3267.9±166.46		80.0±70.14		<0.001

*: Data are given as mean ± SD. Baseline refers to measures obtained at the pre-intervention assessment. P_a: Paired t test was used for analysis to compare the same group before and after intervention. P_b: denotes differences between the 2 groups using an independent t-test.

Table 4. Relationship between dietary antioxidants, dTAC and serum inflammatory markers*

Δ	SOD (ng/mL)	IL-1β (pg/mL)	TGF-β (pg/mL)	TAS (µmol/L)	TOS (µmol/L)	OSI
T-ORAC (µmol TE)	r= -0.104	r= -0.140	r= -0.118	r= -0.272	r= -0.341	r= -0.198
H-ORAC (µmol TE)	r= -0.120	r= -0.108	r= -0.120	r= -0.249	r= -0.337	r= -0.193
TEAC (mmol TE)	r= -0.052	r= -0.151	r= -0.093	r= -0.255	r= -0.338	r= -0.177
TRAP (mmol TE)	r= -0.079	r= -0.187	r= -0.072	r= -0.198	r= -0.315	r= -0.195
FRAP-1 (mmol)	r= 0.146	r= 0.013	r= 0.004	r= 0.244	r= 0.055	r= 0.001
FRAP-2 (mmol)	r= -0.119	r= -0.022	r= -0.122	r= -0.264	r= -0.379*	r= -0.219
FRAP-3 (mmol)	r= -0.063	r= -0.224	r= -0.090	r= -0.260	r= -0.366*	r= -0.188
FRAP-4 (mmol)	r= 0.147	r= -0.083	r= -0.042	r= -0.348	r= -0.106	r= -0.171
DTAC (mg/100g)	r= 0.062	r= -0.265	r= 0.040	r= -0.100	r= 0.112	r= -0.025
TP (µmol TE/100g)	r= -0.091	r= -0.010	r= -0.081	r= -0.229	r= -0.385*	r= -0.246

*: r, Pearson correlation coefficient. T-ORAC:Total Oxygen Radical Absorption Capacity, L-ORAC: Lipophilic Oxygen Radical Absorption Capacity, H-ORAC: Hydrophilic Oxygen Radical Absorption Capacity, TEAC: Trolox Equivalent Antioxidant Capacity, TRAP; Total Radical-Trapping Antioxidant Parameter, FRAP: Ferric Reducing Antioxidant Potential, DTAC: Dietary Total Antioxidant Capacity, TP:Total phenolic

There were no significant changes in TAS or SOD levels in this study. There was also a significant negative correlation between the difference in TOS scores and the difference in TP values. However no significant relationship was found between TAS and TP. Whereas in a study on fats, a positive relationship was found between TAS results and TP [18].While two recent studies with 12 and 6 weeks of grape juice supplementation revealed no

impact [28, 29], several investigations have clearly demonstrated that 2 weeks of grape juice supplementation was adequate to boost blood antioxidant capacity in healthy people [30, 31]. The discrepancies in the study results would also be caused by changes in assessments of plasma antioxidant activity, in addition to the type of intervention and other various study settings [28, 29]. In order for black mulberry anthocyanins to show antioxidant properties, they must not be lost after ingestion and converted into biologically active metabolites after absorption. Parameters such as plasma TAS regress to normal levels immediately after antioxidant supplementation [30, 32]. Therefore, since TAS analysis was not performed immediately after the last black mulberry concentrate intake, TAS levels may have reached normal levels at the time the plasma samples were taken. This situation can be considered as one of the factors affecting the effect of black mulberry concentrate on TAS. In addition, mulberries were stored at -18°C until the concentrate production stage. Antioxidant capacity was not measured again during the study, but it is stated in the literature that there may be losses in the freezing stage. In one study, after 6 months of storage of blueberries at –18°C, an average of 16.7% decrease in total antioxidant content was detected. In this study, there may be losses in antioxidant capacity during the storage phase [33].

The anti-inflammatory effect was also evaluated. In one study, black currant supplementation was found to reduce TNF-α and IL-1β secretion in obese mice fed a high fat/high cholesterol diet [34]. Another study found that obese mice fed mulberry and cherry anthocyanins had lower MDA production, higher SOD and glutathione peroxidase activities, and lower TNF-α, IL-6, and NF-κB expressions. However, no significant change was observed in the IL-1β levels [35]. In an animal study, anthocyanin treatment inhibited age-related cognitive decline in aged mice injected with D-galactose. In the anthocyanin-treated group, the releases of COX2, TGF-β1, and IL-1 cytokines were decreased. Glutathione peroxidase and SOD levels in serum and brain tissue increased in the anthocyanin group, while there was no significant change in the MDA level, except for the decrease in the hippocampus region [36]. In another animal study, after administering *Morus nigra* fruits, a decrease in malondialdehyde levels in the serum and liver and a significant increase in superoxide dismutase, catalase, glutathione peroxidase activities, and strong antioxidant activity were detected compared to the control group [37].

Supporting evidence from clinical trials is also available. In a 24-week randomized controlled trial involving individuals aged 60–80 years with mild cognitive impairment or cardiometabolic risk, daily supplementation with 320 mg anthocyanins significantly reduced LDL cholesterol, CRP, and inflammatory cytokines including IL-6 and IL-1β compared with placebo, with more pronounced effects observed in participants with higher baseline inflammation [46]. In our study, although black mulberry consumption significantly affected TOS and the oxidative stress index, no significant changes were observed in serum inflammatory markers. This may indicate that the antioxidant effects of black mulberry have a limited impact on inflammatory response. Possible explanations include the relatively short duration of the intervention, which may have been insufficient to induce measurable changes in inflammatory parameters. Furthermore, as noted in the literature, the predominance of anti-inflammatory evidence derived from experimental animal studies may contribute to the discrepancies observed between animal and human trials.

Drugs have a more specific, faster, and stronger effect by blocking the stages of the symptoms caused by the disease. Phytonutrients in food, on the other hand, do not have a one-dimensional mechanism of action like drugs but act by changing gene expression in the nucleus at the level of the cell control center [38]. For example, there is evidence that anthocyanins are potent inhibitors of NF-κB, which plays a vital role in inflammation [38]. In other words, phytonutrients aim for a slower but more fundamental change. Therefore, it is important to keep the research period much longer in studies on phytonutrients in terms of obtaining clearer data. 12 weeks may be a short time to see the possible benefits of black mulberry concentrate. In order to better determine the effects of anthocyanins on plasma antioxidant, inflammation, and oxidative stress indicators, it would be beneficial to conduct clinical studies in which different application times and different application doses would be implemented.

In this study, mean BMI was over 25 kg/m^2. During the study, there was no significant change in the weights of individuals in either the intervention or control groups. It is important for the elderly and especially Alzheimer's disease patients to have a BMI value of over 25 kg/m^2 in terms of maintaining sufficient nutrient stores to cope better with diseases or the acute effects of diseases and enabling them to have a faster recovery process compared to the elderly who are of normal weight or underweight has been reported [40]. Weight loss is common in elderly people with dementia, especially those with Alzheimer's disease, but the fact that the Alzheimer's patients participating in this study are still in the early stages may be the reason why they did not experience significant weight loss for 12 weeks. A study reported the reference handgrip strength values over 60 years old, as 33 kg for men and 22 kg for women [41]. In this study, the hand grip strength of both men and women was lower than the reference values. This condition may be associated with dementia. In elderly with sarcopenia, a decrease in upper middle arm circumference is accepted as an indicator of decreased muscle protein stores [42].

There were some limitations to this study. First, it's possible that the study's time frame was insufficient for detecting cognitive changes. Second, unforeseen storage circumstances may have caused a drop in the anthocyanin content and antioxidant capacity of mulberry concentrates. Furthermore, the study results cannot be applied to individuals with early onset dementia and non-dementia Alzheimer's since this study only recruited patients with AD. Finally, the study's small sample size was another drawback, thus a larger and longer follow-up

study is needed to find out how black mulberry affects dementia patients' cognitive function. In addition, conducting the study with a larger sample size could increase the statistical power and the reliability of the results. In the study, the potential loss of anthocyanins during storage was taken into consideration; however, the antioxidant capacity of the concentrates was not remeasured throughout the study. This is another limitation of the study. In future studies, it would be beneficial to regularly measure the antioxidant capacity of the concentrate during the intervention period.

CONCLUSION

In conclusion, the effect of black mulberry (*Morus nigra*) on antioxidant capacity and inflammation was found to be limited in this study. These findings could additional contribution to population-based prevention efforts and clinical interventions for AD. Since the cellular changes related to Alzheimer's disease may occur many years before the appearance of clinical symptoms, individuals in all age groups should consume adequate amounts of vegetables and fruits with high polyphenol content every day. High anthocyanin containing fruits such as black mulberry, which cannot be available in all seasons, may be consumed in concentrated form under appropriate conditions in which antioxidant losses are minimized and 5-Hydroxymethylfurfural (HMF) formation is prevented as much as possible.

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