Original Research Article

Protective Effects of *Medicago sativa* Extract on Oxidative Stress and Apoptosis in a DMBA-Induced Experimental Breast Carcinogenesis Model

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Baran Demir[1], Mehmet Tolga Kafadar[2], Eda Yıldızhan[3], and Murat Akkuş[4]

Abstract

Background: Breast cancer is a leading cause of cancer-related morbidity and mortality worldwide, and oxidative stress plays a critical role in early mammary carcinogenesis. *Medicago sativa* has been reported to possess antioxidant properties; however, its effects on early oxidative stress–apoptosis interplay in breast tissue remain unclear. **Methods:** Twenty-eight female Wistar albino rats were randomly allocated into four groups: control, DMBA, *Medicago sativa*, and DMBA + *Medicago sativa* (n = 7 each). Breast carcinogenesis was initiated by a single intraperitoneal dose of 7,12-dimethylbenz[a]anthracene (DMBA; 80 mg/kg). *Medicago sativa* extract was administered orally at 250 mg/kg/day for 10 weeks. Serum malondialdehyde (MDA), total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) were measured. Mammary tissues were evaluated histopathologically and immunohistochemically for Bax and Bcl-2 expression. **Results:** DMBA administration significantly increased serum MDA, TOS, and OSI levels compared with the control group ($p < 0.05$). *Medicago sativa* treatment significantly reduced MDA, TOS, and OSI levels in the DMBA + *Medicago sativa* group ($p < 0.05$). No statistically significant difference in TAS levels was observed between the DMBA and DMBA + *Medicago sativa* groups. Histopathological analyses revealed moderate fibrosis, inflammatory infiltration, degenerative epithelial changes, and ductal hyperplasia in the DMBA group, whereas these alterations were attenuated following *Medicago sativa* treatment. The immunohistochemical evaluation demonstrated that, compared with the DMBA group, Bax expression with pro-apoptotic function increased, while Bcl-2 expression with anti-apoptotic function decreased in the DMBA + *Medicago sativa* group. **Conclusions:** *Medicago sativa* mitigates DMBA-induced early mammary tissue damage by reducing oxidative stress and modulating Bax/Bcl-2–mediated apoptotic signaling prior to overt tumor formation, highlighting its mechanistic relevance in targeting early stages of breast carcinogenesis.

Keywords

breast cancer, *Medicago sativa*, DMBA, oxidative stress, apoptosis, Bax, Bcl-2

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[1]Department of General Surgery, Tunceli State Hospital, Tunceli, Turkey
[2]Department of General Surgery, Faculty of Medicine, Dicle University, Diyarbakir, Turkey
[3]Department of Histology and Embryology, Faculty of Medicine, Recep Tayyip Erdoğan University, Rize, Turkey
[4]Department of Histology and Embryology, Faculty of Medicine, Dicle University, Diyarbakir, Turkey

Corresponding author:
Mehmet Tolga Kafadar Department of General Surgery, Faculty of Medicine Dicle University Diyarbakir 21000, Turkey
Email: drtolgakafadar@hotmail.com

1. Introduction

In recent years, breast cancer has surpassed lung cancer to become the most common type of cancer worldwide.[1] Due to its high morbidity and mortality rates, it represents a significant health problem for both women and healthcare systems across countries.[2] According to the World Health Organization (WHO), the incidence of breast cancer in 2022 was estimated at 2.29 million, and with 666,103 deaths in the same year, it was the leading cause of cancer-related mortality among women.[3]

In recent years, significant progress has been made in the treatment of cancer with respect to surgical and oncological principles. Alongside these advancements, the effects of medicinal plants on cancer treatment have also emerged as a subject of increasing interest. Within this context, the term *nutraceutical* was first introduced in 1989 by Stephen DeFelice, combining the words *nutrition* and *pharmaceutical*. Since its introduction into the literature, numerous plant-based food products have been studied for their preventive and protective roles against breast cancer. These studies have demonstrated that such products are effective not only in cancer prevention but also in reducing recurrence after treatment.[4,5]

Lignans, coumarins, ascorbic acid, flavonoids, phytoestrogens, and sterols are important agents that can be utilized as phytotherapeutics. These bioactive phytochemicals have been shown to inhibit oxidative stress, suppress inflammation, and modulate critical signaling pathways in cells through apoptosis and neovascularization. As a result, they can eliminate tumor cells *in vitro* and prevent cancer development, while *in vivo* studies have demonstrated their inhibitory effects on tumor growth.[6] In this regard, alfalfa (*Medicago sativa*) has been used in the treatment of various diseases, and its pharmacological effects have recently been observed.[7] In recent years, studies have reported the antioxidant and antineoplastic properties of *Medicago sativa*.[8,9] Moreover, numerous other studies have shown that *Medicago sativa* exhibits anticancer properties through its antioxidant activity and has protective effects against coronary artery disease, osteoporosis, diabetes mellitus, and gastric ulcers.[10,11]

Polycyclic aromatic hydrocarbons (PAHs) were first identified as molecules capable of inducing malignancy in 1921. Subsequent studies demonstrated their role particularly in the initiation of carcinogenesis.[12] They exert their effects by damaging the immune system, causing immunosuppression and immunotoxicity, and generating free radicals.[13] Among these, 7,12-dimethylbenz[a]anthracene (DMBA) is a significant carcinogenic compound.[14] It has been widely used to induce cancer in various experimental models of breast and skin cancer. Its effect is indirect and occurs through metabolism by cytochrome P450 enzymes, leading to the formation of diol epoxides and reactive oxygen species that cause cellular damage.[15] These metabolites exert their carcinogenic activity by disrupting the structure of DNA through various oxygen radicals and inducing lipid peroxidation.[16]

The aim of this study was to investigate the protective effects of *Medicago sativa* leaf extract on a DMBA-induced breast cancer model in rats. Oxidative stress and antioxidant status (TOS, TAS, and MDA), histopathological and immunohistochemical changes, and apoptotic processes (Bax and Bcl-2 expression) were evaluated.

2. Materials and Methods

2.1. Induction of Experimental Breast Cancer

In experimental breast cancer models, 7,12-dimethylbenz[a]anthracene (DMBA) and N-nitroso-N-methylurea (NMU) are commonly used chemical carcinogens. DMBA has well-documented carcinogenic and immunosuppressive properties and has been widely used to induce mammary tumors in rodents.[17,18] Therefore, DMBA was selected for breast cancer induction in this study.

DMBA (Sigma-Aldrich, Darmstadt, Germany) was administered intraperitoneally at a single dose of 80 mg/kg, following the experimental protocol described by Gülbahçe Mutlu et al. Tumor development has been reported to occur approximately 50–140 days after DMBA administration. Animals were monitored throughout the experimental period for clinical signs and pathological changes.[19-21]

2.2. Preparation and Administration of Medicago sativa Extract

The *Medicago sativa* plant used in this study was collected from a village at an altitude of 1300–1400 m in Elazığ, Turkey, and its taxonomic identification was confirmed by Tonçer, a researcher from the Department of Organic Agriculture. The preparation of the plant extract was based on the method described by Kartal et al[22] The removal of the solvent from the crude extract was carried out using a Heidolph rotary evaporator (catalog no: HEID_31011, Schwabach, Germany) at the Pharmacognosy Research Laboratory of Dicle University Faculty of Pharmacy. This process yielded the purified *Medicago sativa* extract. Prior to administration, the extract was reconstituted in distilled water. The extract was prepared at an appropriate concentration to deliver a dose of 250 mg/kg/day, and the gavage volume was adjusted not to exceed 1 mL per

100 g of body weight. Oral administrations were performed using appropriately sized stainless-steel, rounded-tip gavage cannulas with single-use syringes.[23,24]

In a study by Raeeszadeh et al *alfalfa* (*Medicago sativa*) ethanol extract was administered to Wistar rats at doses of 250, 500, and 750 mg/kg, and it was observed that the 250 mg/kg dose exhibited significant antioxidant activity. This dose was therefore selected as the starting dose for the experimental design of the present study.[25]

2.3. Experimental Animals and Study Design

A total of 28 female Wistar albino rats (200–250 g) were included in the study. Animals were housed in standard laboratory cages under controlled environmental conditions (22 ± 3 °C, 12 h light/dark cycle) with free access to standard chow and water. Animals were monitored daily for general health, physical appearance, and behavioral changes. Body weights were recorded regularly, and treatment doses were adjusted accordingly.

The primary aim of this study was to evaluate the potential protective and antioxidant effects of *Medicago sativa* extract in an experimental breast cancer model induced by DMBA. The study design was planned to compare the biochemical and histopathological outcomes of the plant extract administered alone and in combination with DMBA. A positive control group receiving a known chemotherapeutic or anti-estrogenic agent was not included, as the objective of the study was not to compare with standard treatments but to investigate the potential effects of *Medicago sativa* within the experimental model.

Animals were randomly allocated into four experimental groups (n = 7 per group):

Group 1 (Control/Sham): Rats received 1 mL of physiological saline orally via gavage.
Group 2 (DMBA): Rats received a single intraperitoneal dose of DMBA (80 mg/kg).
Group 3 (*Medicago sativa*): Rats received *Medicago sativa* extract orally via gavage at a daily dose of 250 mg/kg.
Group 4 (DMBA + *Medicago sativa*): Rats received a single intraperitoneal dose of DMBA (80 mg/kg), followed 24 h later by oral administration of *Medicago sativa* extract (250 mg/kg/day).

At the end of the 10-week experimental period, in accordance with ethical principles and approved protocols, all rats were administered general anesthesia prior to the initiation of procedures. General anesthesia was induced by intraperitoneal administration of ketamine hydrochloride at a dose of 90 mg/kg (Ketalar; Pfizer, Istanbul, Turkey) and xylazine at a dose of 10 mg/kg (Rompun; Bayer, Istanbul, Turkey). The depth of sedation was regularly monitored at predefined intervals by assessing responses to skin or toe pinch stimuli.

2.4. Sample Collection

Collected blood samples were centrifuged at 3000 rpm for 10 min, and serum was separated and stored at −80 °C until biochemical analyses. Randomly selected mammary tissue samples were fixed in 10% neutral buffered formalin and transferred to the Histology/Embryology Laboratory for histopathological and immunohistochemical analyses.

2.5. Biochemical Analyses

Serum samples were used to evaluate oxidative stress parameters, including malondialdehyde (MDA), total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI).

2.5.1. Malondialdehyde (MDA) Assay

MDA levels, a major secondary product of lipid peroxidation in cell membranes, were determined using the thiobarbituric acid reactive substances (TBARS) method. The principle of the method is based on the reaction of MDA with thiobarbituric acid (TBA) under acidic conditions (pH 3.4) at high temperature, resulting in the formation of a pink-colored MDA–TBA complex. For this purpose, serum samples were mixed with TBA reagent in an oxygenated acidic environment and incubated at 95 °C. After incubation, the absorbance of the resulting colored complex was measured spectrophotometrically at 532 nm. MDA concentrations were calculated based on the obtained absorbance values.[26,27]

Quantitative analysis of serum MDA levels was also performed using a commercial ELISA kit. Rat-specific ELISA kits (Cat. No: E0156Ra) were obtained from Bioassay Technology Laboratory (Shanghai, China) and used according to the manufacturer's instructions. Measurements were carried out using a microplate reader, and the results were calculated based on the standard curve and expressed as nmol/mL.

2.5.2. Total Antioxidant Status (TAS)

Venous blood samples were collected from rats in all experimental groups into EDTA-containing tubes and centrifuged at 3,000 rpm for 10 minutes at +4 °C. The separated plasma samples were stored at −80 °C until analysis. Prior to analysis, both the kit reagents and plasma samples were allowed to reach room temperature.

Total antioxidant status (TAS) levels were measured using a commercial kit from Rel Assay Diagnostics (Gaziantep, Turkey) according to the manufacturer's instructions. Absorbance measurements were performed spectrophotometrically using a Beckman Coulter AU5800 biochemical autoanalyzer. Results were expressed as μmol Trolox equivalent per liter (μmol Trolox Equivalent/L). The spectrophotometric measurement wavelength used for TAS analysis was set at 660 nm.[28]

2.5.3. Total Oxidant Status (TOS)

Serum total oxidant status (TOS) levels were determined using a commercial kit from Rel Assay Diagnostics (Gaziantep, Turkey) according to the manufacturer's protocol. Measurements were performed spectrophotometrically using a Beckman Coulter AU5800 biochemical autoanalyzer.

The analysis is based on the colorimetric method developed by Erel and Erdemli. In this method, oxidant molecules present in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ion; the resulting ferric ion forms a colored complex with xylenol orange, and the intensity of the color change is directly proportional to the total oxidant level of the sample. Results were expressed as μmol H_2O_2 equivalent per liter (μmol H_2O_2 Equivalent/L). The spectrophotometric measurement wavelength used for TOS analysis was set at 532 nm.[29,30]

2.5.4. Oxidative Stress Index (OSI)

To evaluate the overall oxidative stress status, the Oxidative Stress Index (OSI) was calculated. The OSI value was obtained using serum total oxidant status (TOS) and total antioxidant status (TAS) levels according to the following formula:

$$\mathrm{OSI} = (\mathrm{TOS} \ / \ \mathrm{TAS}) \times 100$$

Prior to calculation, TOS results were standardized in μmol H_2O_2 equivalent/L and TAS results in μmol Trolox equivalent/L. The OSI values were then converted to percentage values and expressed as arbitrary units (AU).[31]

2.6. Histological and Histochemical Analyses

Mammary tissue samples were fixed in 10% formalin for 24 h, washed overnight under running tap water, and embedded in paraffin. Sections of 5 μm thickness were obtained using a microtome (Leica RM2265, Wetzlar, Germany) and mounted on positively charged slides.

Hematoxylin and eosin (H&E) staining was performed to evaluate histopathological changes. Vascularization, inflammatory cell infiltration, apoptotic-degenerative changes, and pleomorphism were semi-quantitatively scored as follows: 0 (none), 1 (very mild, 1–15%), 2 (mild, 16–30%), 3 (moderate-low, 31–45%), 4 (moderate, 46–60%), 5 (severe, 61–75%), and 6 (very severe, 76–100%).[32]

2.7. Immunohistochemical Analysis

Apoptosis plays a crucial role in cell proliferation under both physiological and pathological conditions. It is a fundamental process in development and maintenance of homeostasis. Pro-apoptotic factors such as Bax and anti-apoptotic factors such as Bcl-2 balance the apoptotic process. In cancer, apoptosis occurs as a result of the activation of caspases, which belong to the cysteine protease family.[33,34] Caspases eliminate molecules that inhibit apoptosis, thereby promoting cell death through the apoptotic pathway.[35] The Bcl-2 family proteins are key regulators of apoptosis, consisting of both pro- and anti-apoptotic members. The likelihood of apoptosis is determined by the Bcl-2/Bax ratio.[36,37]

Immunohistochemical staining was performed on randomly selected mammary tissue sections (5 μm) to evaluate apoptotic activity. Sections were stained with primary antibodies against Bax and Bcl-2 to assess pro-apoptotic and anti-apoptotic protein expression. The balance between Bax and Bcl-2 expression was used to evaluate apoptosis-related changes in mammary tissues.

Immunohistochemical evaluation was performed under a light microscope by two independent observers blinded to the experimental groups. The intensity and distribution of immunopositive staining were assessed semi-quantitatively.

The reporting of this study conforms to ARRIVE 2.0 guidelines.[38]

2.8. Statistical Analysis

Sample size calculation was performed using G*Power software (version 3.1.9.4). Based on Cohen's criteria, an effect size of 0.70, $\alpha = 0.05$, and power = 0.80 were used, resulting in a minimum sample size of 28 animals. Statistical analyses were conducted using SPSS for Windows (version 20.0; IBM Corp., Armonk, NY, USA).

Data normality was assessed using the Shapiro–Wilk test, skewness and kurtosis values, and Q–Q plots. Normally distributed data were analyzed using one-way analysis of variance (ANOVA), whereas non-normally distributed data were analyzed using the Kruskal–Wallis H test. Pairwise comparisons were performed using the Mann–Whitney U test where appropriate. Results were expressed as mean ± standard error (SE), and a p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of Medicago sativa on Oxidative Stress Parameters

3.1.1. Serum Malondialdehyde (MDA) Levels

Serum malondialdehyde (MDA) levels differed significantly among the experimental groups. The highest mean MDA level was observed in the DMBA group, whereas significantly lower levels were detected in the DMBA + *Medicago sativa* group ($p < 0.05$). No statistically significant difference was found between the *Medicago sativa* and DMBA + *Medicago sativa* groups ($p > 0.05$). These findings indicate that *Medicago sativa* administration attenuated DMBA-induced lipid peroxidation.

3.1.2. Total Antioxidant Status (TAS)

Analysis of total antioxidant status (TAS) revealed no statistically significant difference between the DMBA and DMBA + *Medicago sativa* groups ($p > 0.05$). However, significant differences were observed between the DMBA + *Medicago sativa* group and both the control and *Medicago sativa* groups ($p < 0.05$). The highest mean TAS value was recorded in the DMBA group, whereas the lowest value was observed in the *Medicago sativa* group.

3.1.3. Total Oxidant Status (TOS)

Total oxidant status (TOS) levels showed significant differences among the groups. The DMBA group exhibited the highest serum TOS levels, while significantly lower values were observed in the DMBA + *Medicago sativa* group compared with the DMBA group ($p < 0.05$).

3.1.4. Oxidative Stress Index (OSI)

The oxidative stress index (OSI), calculated as the ratio of TOS to TAS, differed significantly between the groups. The DMBA group demonstrated the highest mean OSI value, whereas the DMBA + *Medicago sativa* group showed a significantly lower OSI value ($p < 0.05$). No significant difference was detected between the control and *Medicago sativa* groups ($p > 0.05$). The results of MDA, TAS, TOS, and OSI analyses for all experimental groups are summarized in Figure 1. Mean±Standard deviations of serum MDA, TAS, TOS and OSI values averaged between groups are shown in Table 1.

3.2. Histopathological Findings

At the end of the experiment, the breast tissues obtained were first evaluated macroscopically, and no visible tumor formation was observed in any of the experimental groups. The tissues were then fixed appropriately for histopathological examination and embedded in paraffin blocks after routine tissue processing. The prepared sections were examined microscopically. In the histopathological evaluation, the parameters of vascularization, inflammatory cell infiltration, apoptotic-degenerative changes, and cellular pleomorphism were scored semi-quantitatively. These parameters were graded according to the severity of the lesion.

Mammary tissue sections from the control group exhibited normal histological architecture. In the *Medicago sativa* group, very mild fibrosis was observed. In contrast, the DMBA group showed moderate fibrosis, inflammatory cell infiltration, degenerative changes in mammary epithelial cells, and ductal hyperplasia. The DMBA + *Medicago sativa* group displayed mild fibrosis and increased vascularization compared with the DMBA group.

Histopathological parameters were scored semi-quantitatively and statistically compared between groups. The analysis revealed a statistically significant difference in histopathological scores between the DMBA group and the DMBA + *Medicago sativa* group ($p < 0.05$).

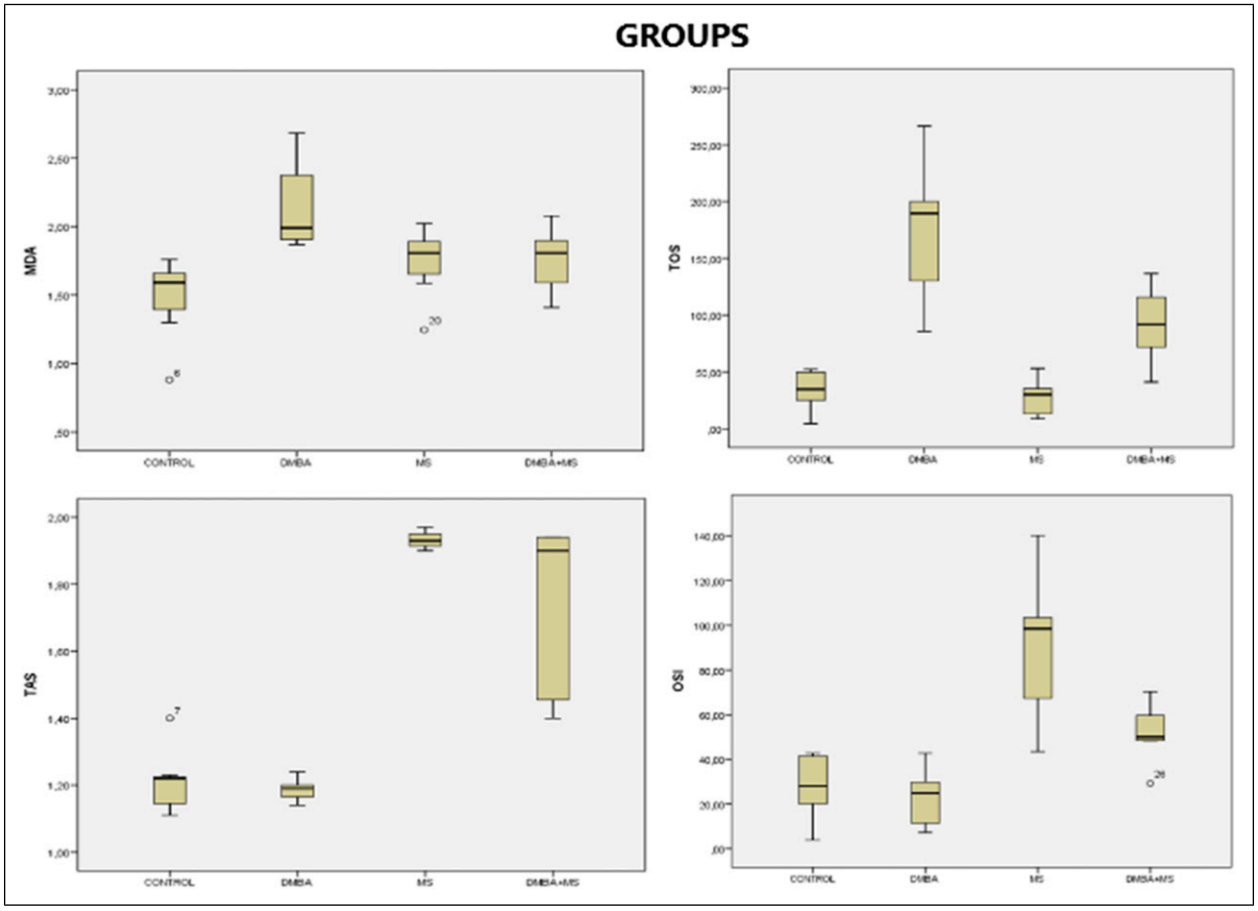

Figure 1. Statistical analysis of MDA, TAS, TOS, and OSI across all groups

Representative histological images are shown in Figure 2.

3.3. Immunohistochemical Findings

3.3.1. Bax Expression

Bax immunoreactivity was very weak in the control group and moderately positive in the *Medicago sativa* group. In the DMBA group, Bax expression was weakly positive to negative, comparable to the control group. In contrast, the DMBA +

Table 1. Mean ± Standard Deviation of Serum MDA, TAS, TOS, and OSI Values Calculated for Each Group

Groups	MDA (nmol/mL)	TAS (μmol)	TOS (μmol)	OSI (AU)
Control	1.47±0.30[b]	1.21± 0.09[b,d]	34.32±1 7.65[b,d]	28.29±14.72[b,d]
DMBA	2.15±0.32[a,c,d]	1.93± 0.02[a,c]	171.68±62.31[a,c,d]	89.11±33.13[a,c,d]
MS	1.73±0.26[b]	1.18± 0.03[b,d]	26.91±16.23[b,d]	22.47±13.06[b,d]
DMBA+MS	1.75±0.23[b]	1.71± 0.26[a,c]	92.05±33.32[a,b,c]	52.27±13.12[a,b,c]

TAS; Total Antioxidant Capacity (μmol H_2O_2 equivalent/L), TOS; Total Oxidant Capacity (μmol Trolox equivalent/L), MDA; Malondialdehyde (nmol/mL).OSI; Oxidative Stress Index (arbitrary unit). DMBA; 7,12-dimethylbenz[a]anthracene, MS; *Medicago sativa* extract.
[a]different from control.
[b]different from DMBA.
[c]different from MS.
[d]different from DMBA+MS.

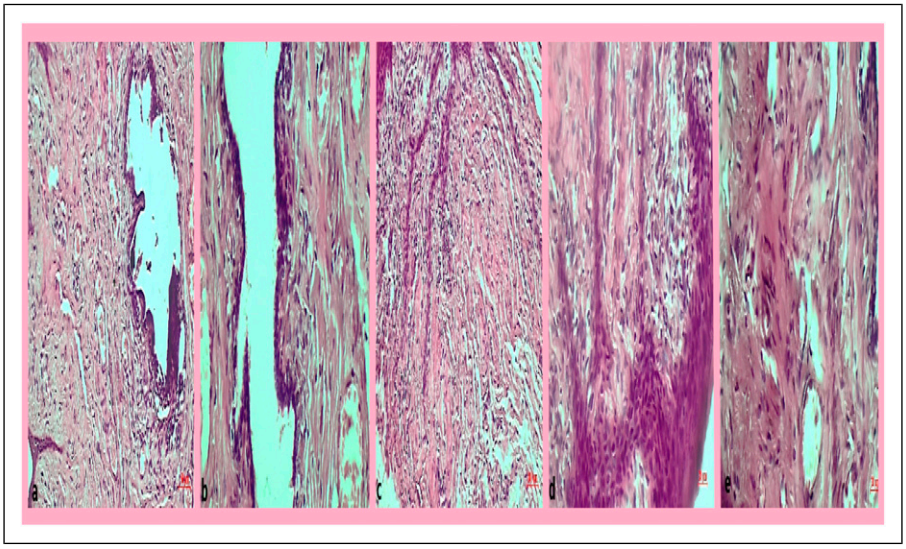

Figure 2. Histopathological Micrographs of Specimens. (A) Control group: Mammary tissue with normal appearance (H&E, Bar 50 µm). (B) MS group: Mild fibrosis (H&E, Bar 50 µm). (C) DMBA group: Inflammatory cell infiltration, moderate fibrosis, pleomorphic cell structure (H&E, Bar 100 µm). (D) DMBA group: Vascularization and degenerative changes in mammary cells (H&E, Bar 20 µm). (E) DMBA+MS group: Mild proliferation and moderate fibrosis (H&E, Bar 20 µm)

Medicago sativa group exhibited stronger Bax positivity compared with the DMBA group, indicating enhanced pro-apoptotic signaling. Representative images are presented in Figure 3.

3.3.2. Bcl-2 Expression

Bcl-2 immunoreactivity was negative to very weak in the control and *Medicago sativa* groups. The DMBA group demonstrated moderately widespread positive Bcl-2 expression in mammary tissues. In the DMBA + *Medicago sativa* group, Bcl-2 expression was overall weakly positive compared with the DMBA group. Representative immunohistochemical images are shown in Figure 4.

4. Discussion

Medicinal and aromatic plants have been used for centuries in the prevention and treatment of various diseases, and scientific interest in their bioactive properties continues to increase. These plants are rich in phenolic compounds, including flavonoids, phenolic acids, and phenolic terpenes, which are particularly abundant in leaves and flowers and are well recognized for their antioxidant and anti-inflammatory activities.[39] Such properties have been suggested to play a role in modulating oxidative stress–related diseases, including cancer.

Oxidative stress is a key contributor to breast carcinogenesis, primarily through lipid peroxidation and subsequent cellular damage. Malondialdehyde (MDA), a major end product of lipid peroxidation, forms cross-links with lipids, proteins, and nucleic acids and serves as a reliable biomarker of oxidative damage.[39-43] Elevated MDA levels have been consistently reported in both breast cancer tissues and serum samples, reflecting impaired tissue oxygenation and increased free radical activity during carcinogenesis.[40,41] Moreover, increased lipid peroxidation has been associated with DNA strand breaks and genotoxic damage in experimental breast cancer models.[42,43] In agreement with these findings, the present study demonstrated the highest serum MDA levels in the DMBA group, whereas *Medicago sativa* co-administration significantly reduced MDA levels. The absence of a significant difference between the MS and DMBA+MS groups suggests that MS effectively counteracted DMBA-induced lipid peroxidation rather than exerting a pro-oxidant effect.[44]

Endogenous antioxidant defense systems play a crucial role in maintaining redox homeostasis and protecting tissues from oxidative injury. Enzymes such as superoxide dismutase and catalase constitute essential components of this defense network, and reduced antioxidant capacity has been reported in various cancer types.[45-47] In the present study, total oxidant status (TOS) was significantly elevated in the DMBA group and markedly reduced following MS treatment, indicating attenuation of systemic oxidative burden. Although total antioxidant status (TAS) did not differ significantly between the DMBA and DMBA+MS groups, this finding should be interpreted with caution. TAS represents a cumulative systemic measure and may not fully reflect early or tissue-specific antioxidant responses during the initial stages of carcinogenesis.

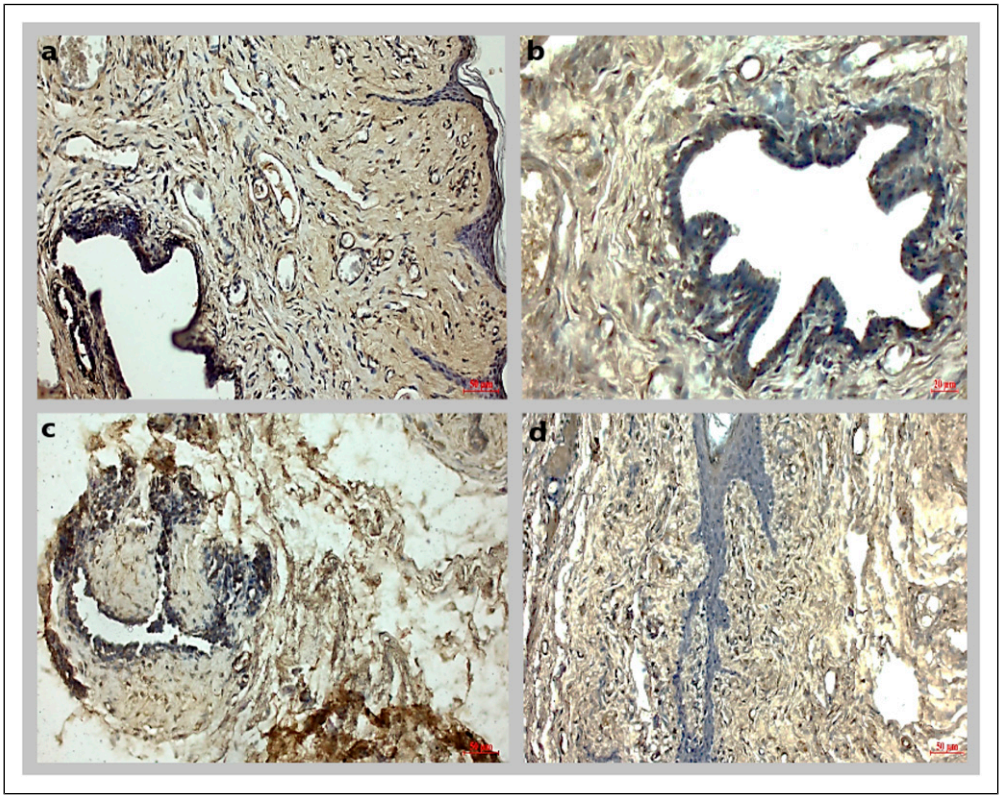

Figure 3. Immunohistochemical Micrographs of Specimens Showing Bax Expression Staining: Bax immunohistochemistry; Counterstain: Hematoxylin; Bar: 50 µm. (A) Control group: Very low level of expression (B) MS group: Moderate level of expression (C) DMBA group: No expression observed (D) DMBA+MS group: Low level of expression

Importantly, the concurrent reductions in MDA, TOS, and OSI strongly support a biologically meaningful antioxidant effect of MS despite the lack of a pronounced TAS difference.

The oxidative stress index (OSI), which integrates both oxidant and antioxidant parameters, provides a more comprehensive assessment of redox balance. In this study, OSI values were highest in the DMBA group and significantly reduced in the DMBA+MS group, further confirming the protective effect of MS against DMBA-induced oxidative stress. The lack of difference between the control and MS groups indicates that MS did not disrupt physiological redox homeostasis under non-stressed conditions.

Histopathological alterations are well documented in DMBA-induced breast carcinogenesis and include inflammatory cell infiltration, epithelial hyperplasia, fibrosis, and early neoplastic changes.[48,49] In line with previous reports, breast tissues from DMBA-treated rats in the present study exhibited moderate fibrosis, inflammatory infiltration, degenerative epithelial changes, and ductal hyperplasia. In contrast, MS administration following DMBA exposure resulted in markedly milder histopathological alterations, suggesting preservation of tissue architecture and suppression of early carcinogenic changes. These findings are consistent with previous studies demonstrating the protective effects of phytochemicals against DMBA-induced mammary tissue damage.[50]

Apoptosis plays a central role in eliminating damaged or transformed cells during carcinogenesis. Members of the Bcl-2 protein family regulate mitochondrial apoptotic pathways by balancing pro-apoptotic and anti-apoptotic signals. Bcl-2 overexpression promotes cell survival and has been associated with tumor progression, whereas Bax acts as a pro-apoptotic mediator whose loss enhances neoplastic potential.[51-53] In the present study, weak or absent Bax expression accompanied by moderate Bcl-2 positivity in the DMBA group reflects suppression of apoptotic signaling during early carcinogenic insult. Notably, MS treatment enhanced Bax expression while reducing Bcl-2 immunoreactivity, indicating restoration of pro-apoptotic balance. These findings suggest that MS may facilitate the elimination of damaged mammary cells through modulation of apoptosis-related pathways.

Despite these findings, several limitations of the present study should be acknowledged. The experimental duration was deliberately limited to 10 weeks to focus on early, pre-neoplastic molecular and cellular alterations induced by DMBA rather than macroscopic tumor formation. Accordingly, the effects of *Medicago sativa* on long-term tumor incidence, growth, and

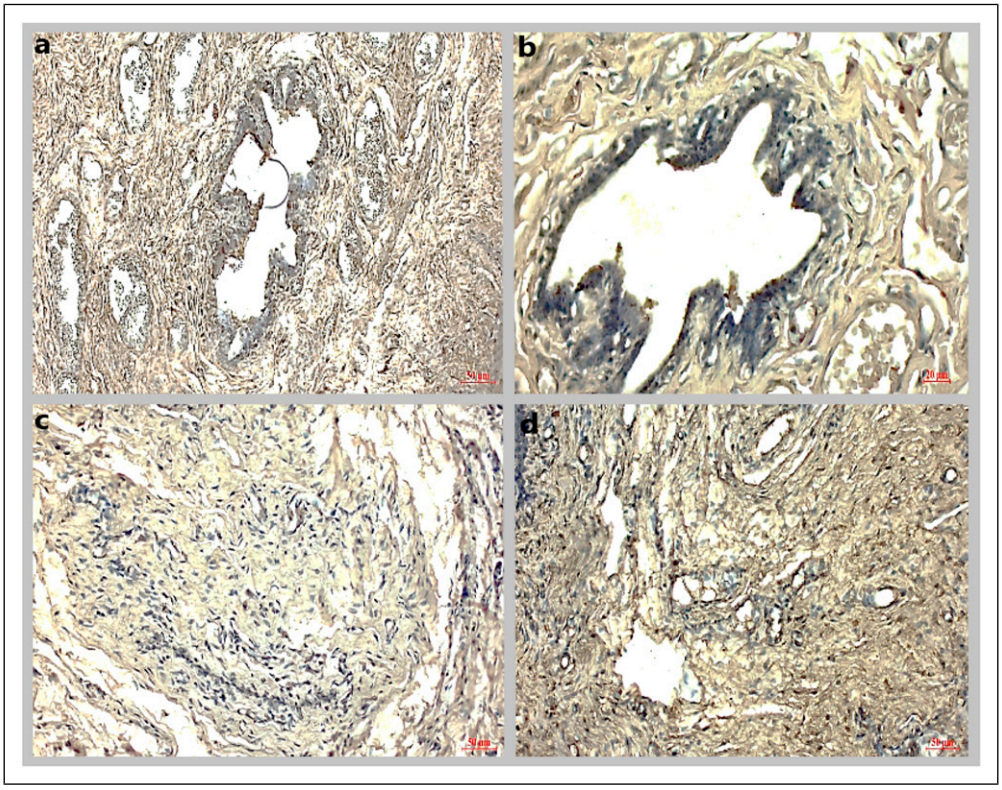

Figure 4. Immunohistochemical Micrographs of Specimens Showing Bcl-2 Expression Staining: Bcl-2 immunohistochemistry; Counterstain: Hematoxylin; Bar: 50 µm. (A) Control group: Very low level of expression (B) MS group: Very low level of expression (C) DMBA group: Moderate level of expression (D) DMBA+MS group: Low-to-moderate level of expression

progression could not be assessed. In addition, total antioxidant status represents a cumulative systemic parameter and may not fully reflect dynamic or tissue-specific redox responses during the early stages of carcinogenesis. In this context, the absence of a statistically significant difference in TAS between the DMBA and DMBA + *Medicago sativa* groups does not negate biologically meaningful antioxidant activity, particularly given the consistent improvements observed in MDA, TOS, OSI, histopathological findings, and apoptosis-related markers. Finally, although Bax and Bcl-2 immunoreactivity provided insight into apoptosis-related signaling, further molecular analyses are warranted to elucidate downstream mechanisms involved in *Medicago sativa*–mediated protection.

5. Conclusions

The present study demonstrates that *Medicago sativa* exerts a protective effect against DMBA-induced early breast tissue injury through coordinated modulation of oxidative stress and apoptosis-related pathways. The consistent reduction in lipid peroxidation and systemic oxidant burden, together with improved histopathological architecture and a shift toward a pro-apoptotic Bax/Bcl-2 profile, indicates that *Medicago sativa* primarily targets early molecular and cellular events preceding overt tumor formation. Importantly, the use of a non-tumor endpoint model allowed the identification of these early mechanistic alterations, highlighting the capacity of *Medicago sativa* to interfere with carcinogenic signaling before irreversible neoplastic transformation occurs. Collectively, these findings provide mechanistic support for the potential of *Medicago sativa* as a complementary strategy aimed at the prevention or early intervention of breast carcinogenesis, warranting further studies to explore its long-term and translational relevance.

Ethical Considerations

All experimental procedures involving animals were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and complied with the ARRIVE guidelines. The study protocol was reviewed and

approved by the Local Animal Ethics Committee of Dicle University Prof. Dr. Sabahattin Payzın Health Sciences Research and Application Center (Approval No: [TIP. 22.028], Date: [01.06.2023])

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ORCID iDs

Baran Demir https://orcid.org/0000-0002-1416-8520
Mehmet Tolga Kafadar https://orcid.org/0000-0002-9178-7843
Eda Yıldızhan https://orcid.org/0000-0002-5648-6498
Murat Akkuş https://orcid.org/0000-0002-1659-1189

Author Contributions

Conceptualisation, B.D.; methodology, B.D. and E.Y.; investigation, B.D., M.T.K., E.Y. and M.A.; writing, original draft preparation, B.D. and E.Y.; writing, review and editing, B.D., M.T.K. and E.Y. All authors have read and agreed to the published version of the manuscript.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supplemental Material

Supplemental material for this article is available online

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